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GRANT NUMBER DAMD17-93-J-3031

TITLE: Membrane Transport: Cellular Probe of Heat Stroke

PRINCIPAL INVESTIGATOR: John S. Willis, Ph.D.

CONTRACTING ORGANIZATION: University of Georgia
Athens, Georgia 30602-2556

REPORT DATE: September 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 2

19971114 044

REPORT DOCUMENTATION PAGE

Form Approved
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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1997	3. REPORT TYPE AND DATES COVERED Final (2 Aug 93 - 1 Aug 97)
4. TITLE AND SUBTITLE Membrane Transport: Cellular Probe of Heat Stroke			5. FUNDING NUMBERS DAMD17-93-J-3031
6. AUTHOR(S) John S. Willis, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Georgia Athens, Georgia 30602-2556			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200) In vitro effects of temperature on permeation of Na ⁺ and K ⁺ were explored in red cells of guinea pig to evaluate the Energy Depletion Hypothesis of heatstroke. Prior to Midterm Report it was found that large changes in cell [Na ⁺] and [K ⁺] do not occur at 41°C and 45°C, that increase in pump activity matches elevated Na ⁺ permeation at 41°C and that steep rise in K-Cl cotransport at 41°C and 45°C accounts for most increase in passive K ⁺ permeation. Since Midterm Report this large temperature effect on K-Cl cotransport has been shown to operate indirectly through regulation of the carrier, most probably the phosphatase that activates it. Increased net loss of K ⁺ through this pathway protects the cell from swelling at 41°C and 45°C. In vitro this loss is balanced by uptake of K ⁺ through the Na-K pump, but if pumps are limited in vivo, it could account for hyperthermic hyperkalemia. Cell acidification (and swelling) causes an enormous increase in Na ⁺ uptake at 41°C and 45°C through an unknown amiloride-sensitive pathway. This devastating rise in Na permeation could, if general, meet the primary condition for cell failure postulated by the Energy Depletion Hypothesis.			
14. SUBJECT TERMS Heat, Heatstroke, Temperature, Membrane Permeability, Sodium, Potassium, Red Blood Cells			15. NUMBER OF PAGES 66
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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John S. Walker Aug. 15, 1987
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TABLE OF CONTENTS

COVER PAGE	i
REPORT DOCUMENTATION PAGE	ii
FOREWORD	iii
INTRODUCTION.....	4
(BODY):	
METHODS AND MATERIALS.....	6
Choice of material.....	6
Collection and handling of blood.....	6
Choice of temperatures for incubation.....	6
Studies of K-Cl cotransport.....	7
Unidirectional influxes; K^+ influx.....	7
K^+ efflux.....	8
Inhibitors of K^+ fluxes.....	8
Analytical methods.....	9
Studies of acid-activated amiloride-sensitive Na^+ influx.....	10
Adjustment of cytoplasmic pH.....	10
Media used for studies of Na^+ influx.....	11
Measurement of Na^+ influx.....	11
Measurement of H^+ efflux.....	12
RESULTS.....	13
Part I. Thermal activation of K-Cl cotransport precludes cell	
cell swelling during hyperthermia.....	13
K^+ influx.....	13
Unidirectional K^+ efflux.....	15
Balance of K^+ influxes and effluxes/elevated temperature..	16
Total K^+ influx vs. total K^+ efflux.....	16
Balance of K^+ flux in absence of K-Cl cotransport.....	17
Change in water content in absence of K-Cl cotransport....	17
Na-K pump K^+ uptake vs. K-Cl cotransport K^+ loss.....	17

Part II. Amiloride-sensitive (AS) Na^+ influx: acidification	
with warming causes explosive rise in Na^+ uptake.....	18
Dependence of AS Na^+ influx on $[\text{Na}^+]_o$	18
Interaction of acid activation and shrinkage activation...	19
Effect of temperature on Type 1 and Type 2 AS Na^+ influx..	20
Dose-inhibition by amiloride and its derivatives.....	20
Effect of cytoplasmic Mg^{2+} on Type 1 and 2 AS Na^+ influx..	21
H^+ efflux coupled to Type 1 and Type 2 AS Na^+ influx.....	21
Net gain of Na^+ vs. Type 2 AS Na^+ influx.....	22
Effect of calyculin on shrinkage-activated AS Na^+ influx..	22
DISCUSSION.....	24
Overview.....	24
The thermal activation of the K-Cl cotransporter.....	24
Mediation of thermal activation.....	25
Physiological significance.....	28
Heat-acid-swelling activated, amiloride-sensitive Na^+ influx...	29
Physiological significance.....	31
CONCLUSIONS.....	32
REFERENCES CITED	34
(APPENDIX):	
Table 1. Delay in change of K^+ influx after a change in temperature.	40
Table 2. Cell water, Cl^- , total Mg^{2+} , ATP and H^+	41
Table 3. Water content of cells with and without calyculin (25 nM) ..	42
Table 4. Comparison of net fluxes of K^+ through the Na-K pump	
and K-Cl cotransporter at 37°C and 41°C.....	43
Table 5. Effect of Mg-depletion on amiloride-sensitive Na^+ influx ..	44
Table 6. Comparison of net change of cell $[\text{Na}^+]$ with unidirectional	
entry of Na^+ under "Type 2" conditions.....	45
Table 7. Summary of two patterns of amiloride-sensitive Na^+ influx .	46

Fig. 1. Effect of temperature on components of K^+ influx.....	47
Fig. 2. Calyculin inhibition of K-Cl cotransport.....	48
Fig. 3. Time-course of effect of rapid increase or decrease in temperature on K^+ influx.....	49
Fig. 4. Effect of temperature on K^+ influx with hydroxylamine.....	50
Fig. 5. Effect of temperature on unidirectional K^+ efflux.....	51
Fig. 6. Effect of temperature on balance of K^+ influx and K^+ efflux.	52
Fig. 7. Ratio of K^+ influx to K^+ efflux in guinea pig red cells in presence of inhibition of K-Cl cotransport at 37°C and 41°C....	53
Fig. 8. Na-dependence of amiloride-sensitive (AS) Na^+ influx in red cells with cytoplasmic acidification.....	54
Fig. 9. Effect of osmolarity on AS Na^+ influx in acidified red cells.	55
Fig. 10. Effect of cytoplasmic pH on amiloride-sensitive Na^+ influx in hypertonically incubated guinea pig red cells.....	56
Fig. 11. Effect of temperature on two types of AS Na^+ influx.....	57
Fig. 12. Amiloride inhibition of two types of Na^+ influx.....	58
Fig. 13. DMA inhibition of two types of AS Na^+ influx	59
Fig. 14. H^+ loss from guinea pig red cells.....	60
Fig. 15. Calyculin on shrinkage-activated AS Na^+ influx.....	61
BIBLIOGRAPHY	62
PERSONNEL.....	63

INTRODUCTION

The purpose of this research project was to explore aspects of cellular regulation of ion content and cell volume that might shed light on the causes of heatstroke brought on by physical exertion in a hot environment. At the level of the whole organism, numerous failures of specific systems have been identified as contributing to heat stroke. The premise of this project was that an understanding of the response of single cells to elevated temperature could play a crucial role in unraveling the complex relationships leading to heatstroke.

The pioneering thinking in this field was done by Dr. Roger Hubbard and his associates at USARIEM and was encapsulated in the Energy Depletion Model (Hubbard *et al.*, 1987; Hubbard and Armstrong, 1988). This hypothesis focused on the Na-K pump, both as a source of heat and as a sink for metabolic fuel. The original purpose of this project was to test certain expectations of the Energy Depletion Model: whether permeation of cations (especially Na^+) increased steeply at elevated temperatures, whether cell $[\text{Na}^+]$ rose with warming and whether the Na-K pump could keep pace with increased Na^+ entry (and K^+ loss). Specific pathways involved in these processes were to be identified and pursued.

For this purpose we have used guinea pig red cells and our results through February 1, 1995 were described in the Midterm Report. Those early results can be briefly summarized as follows: Warming did cause a rise in Na^+ influx at 41°C and 45°C relative to 37°C , though not an unduly steep one. There was no rise in cell Na^+ concentration at the higher temperatures in two hours, but a tendency existed for a rise at longer time intervals (3 hours) and at higher temperature (45°C). The Na-K pump kept pace with Na^+ entry at 41°C but was numerically somewhat slower than Na^+ entry at 45°C . Relative rise in passive influx of K^+ corresponded with that for Na^+ .

Thus, while the results of the first half of this study confirmed that the Na-K pump and Na^+ leak were elevated by warming, they did not support

any unusual contribution to cell metabolism or energy depletion from this source. However, when we investigated the effects of warming on the *components* of Na^+ and K^+ influx, patterns emerged that were relevant to hyperpyrexia and heatstroke in a way independent of the Energy Depletion Model: (1) The ability to elicit increase in Na-H exchange by cell shrinkage *decreased* with warming (Fig. 5 of Midterm Report). (2) Influx of K^+ through a diffusive leak pathway was little affected by warming and Na-K-Cl cotransport actually *declined* with warming (Fig. 7 of Midterm Report) and also became nonresponsive to cell shrinkage (i.e., could no longer be activated by hypertonic incubation, Fig. 8 of Midterm Report). (3) The pathway that accounted for most of the rise in K^+ influx above 27°C was the K-Cl cotransport pathway (Fig. 7 of Midterm Report).

In the Midterm Report seven unresolved questions were posed for further investigation in the second half of the grant period. Of these seven questions, four have now been effectively addressed and are the main subject of this Final Report. Those four questions are: Does the large activation of K-Cl cotransport by warming represent an effect of temperature on regulation of the carrier (e.g., dephosphorylation by phosphatase), and, if so, is the decrease in Na-H transport coordinately regulated? Is increased K^+ efflux at elevated temperature balanced by the Na-K pump? Does increased acidity at higher temperatures contribute to greater leakiness of the plasma membrane for Na^+ ? Does Na-K-Cl cotransport play a role in modulating ion balance at higher temperatures?

For purposes of experimental attack, these issues were grouped into two main foci in the second half of the project: (1) following up on the findings related to thermal activation of the K-Cl cotransporter and (2) investigation of acid-activated, amiloride-sensitive entry in red cells. Accordingly the subsequent sections in this report are organized to reflect this dual thrust.

METHODS AND MATERIALS

Choice of material. Guinea pig red cells were chosen for this study both because the guinea pig has long been a standard animal model with constantly maintained normal mammalian body temperature and because transport of Na^+ and K^+ in guinea pig red cells has been well studied. In particular, their Na-K pump was been characterized (Ellory & Willis, 1982; Willis & Ellory, 1983; Marjanovic & Willis, 1992), and they have been reported to possess Na-K-Cl cotransport (Hall and Willis, 1984), K-Cl cotransport (Hynes and Willis, 1987) and Na-H exchange (Zhao and Willis, 1993).

Collection and handling of blood. Blood was drawn by heart puncture into a heparinized syringe from guinea pigs that had been anesthetized with xylazine and ketamine. The animal was then killed while it was still under deep anesthesia by cutting the spinal cord and severing the carotid arteries. The procedures used in this study were approved by the Animal Use Committee of the University of Georgia.

The blood was diluted with simple incubation medium (150 mM-NaCl, 5 mM-KCl, 10 mM-glucose, 5 mM-adenosine, MOPS buffer, 10 mM, pH 7.4 and 0.1 mM EDTA), centrifuged and the 'buffy coat' of white cells removed. The red cells were then washed twice more in the same way, resuspended in the same medium to a 'hematocrit' of about 10 per cent and held in ice until use.

Choice of temperatures for incubation. The rectal temperature of 5 guinea pigs was $37.4 \pm 0.2^\circ\text{C}$. The main focus of this study was elevated temperatures. Accordingly 37°C was used as a 'base' or control temperature, 41°C as a moderately elevated temperature representing the limits achieved in fever or exertion hyperthermia, and 45°C as an egregiously high temperature. Since the K-Cl cotransporter appeared in preliminary studies to be operational at 37°C , lower temperatures (32°C or 27°C) were also used in some cases to encompass a full range of activation. Samples of cell suspension (1 ml in 1.5 ml microcentrifuge tubes, 3-5 per cent hematocrit)

were suspended in separate water baths for each temperature used and were allowed to equilibrate a minimum of 5 minutes before flux determinations were initiated.

Studies of K-Cl cotransport

Unidirectional influxes

K⁺ Influx. For determination of K⁺ influx ⁸⁶Rb (in the presence of 5 mM nonradioactive KCl) was used in most experiments except as noted below. Influx was initiated after temperature equilibration by rapid mixing of the suspension with 20 ul of solution containing the radioactive isotope. After incubation with isotope for 15-20 min, each sample of cells (about 30 ul) was washed 3-4 times in 1.2 ml ice-cold isotonic, buffered Na-free medium (107 mM MgCl₂, 10 mM Tris, pH 7.8) using short (15 sec, 13,000 g) spins in a microcentrifuge to remove excess isotope. Cells were lysed in 5 per cent trichloroacetic acid and the precipitate removed by centrifugation; the supernatant was diluted to 4 ml with water and its radioactivity counted in a liquid scintillation counter. Computation of influx was based on specific activity determined from a suitable standard made from stock isotope and on cell volume computed from absorption of hemoglobin at 540 nm of the suspension related to measured hematocrit.

In experiments where temperature was instantly changed, 10-15 ml of cell suspension was incubated in 20 ml glass centrifuge tubes. Initial samples were taken at 2 min intervals for 6 min for determination of initial rate of ⁸⁶Rb uptake. Part of the suspension was then removed to a clean, dry, temperature-equilibrated tube and diluted with an equal volume of warmer or colder medium whose temperature was predetermined to achieve the desired temperature after rapid mixing. This diluting medium was identical in every way to the original suspension medium (including specific activity of ⁸⁶Rb). Any effect caused by decrease of hematocrit resulting from this dilution was controlled for in parallel suspensions of

cells that were diluted at the same time with medium of the same temperature.

In one series of experiments, K^+ influx was measured in the same samples of cells as were used to measure K^+ efflux. This was accomplished by incubating the cells in K-free medium with 5 mM nonradioactive RbCl replacing KCl. To be parallel with K^+ efflux (see below), cells were collected at 5 minutes and at a later time (20 - 25 min), their Rb content determined by emission flame photometry and influx computed from the Rb^+ uptake between the two times. The cells were otherwise handled as in the radioactive isotopic experiments.

K^+ efflux. In order to avoid a long period of preincubation required for loading cells with radioisotope, unidirectional efflux of K^+ was measured simply as loss of K^+ from cells into a K-free medium containing nonradioactive RbCl as a replacement for KCl. To do this, cell suspension made with a K-free medium with 5 mM RbCl, was introduced to the microcentrifuge tubes containing the same medium for incubation, samples were removed at timed intervals, centrifuged and the supernatant was drawn off. An aliquot of the supernatant was taken for analysis by flame emission photometry. Another aliquot was taken for estimation of hemoglobin either by Bradford assay (Bradford, 1976) or by measurement of absorption at 540 nm. In experiments with calyculin and okadaic acid, requiring introduction of inhibitor in an ethanol carrier, controls were run with the same concentration of ethanol (0.5 per cent final concentration), and 0.5 per cent albumin was also included in the medium to reduce lysis. Efflux was calculated from the loss of K^+ to the medium between an initial sample taken at 5 min and a later sample taken after 20 - 25 min of incubation, corrected for K^+ loss due to lysis.

Inhibitors of K^+ fluxes. Except where noted, cells were incubated in 100 μ M ouabain to eliminate the component of influx through the Na-K pump (Willis & Ellory, 1983). To differentiate fluxes through the Na-K-Cl

cotransport system, the inhibitor of Na-K-Cl cotransport, bumetanide, was used. In trial studies with cells incubated in hypertonic medium (to minimize K-Cl cotransport and maximize Na-K-Cl cotransport), 10 μ M bumetanide was found to be maximally inhibitory to K^+ influx, and in cells incubated in hypotonic medium (with maximized K-Cl cotransport) the same concentration caused no greater inhibition of influx than that expected for inhibition of Na-K-Cl cotransport. Furthermore, in the presence of 10 μ M bumetanide there was no Na-dependent component of difference in K^+ influx between hypertonic and hypotonic media.

As an inhibitor for K-Cl cotransport, in the presence of ouabain and bumetanide, hypertonic incubation was used. Media were made hypertonic by the addition of sucrose to an osmotic concentration of 450 mOsm. To verify that this was a sufficiently high osmolarity to ensure a full inhibition of K-Cl cotransport at all the temperatures used, trials were run of K^+ influx vs. osmolarity at 37°C - 45°C. The results showed that while there may be a shift toward higher osmolarities at higher temperatures for osmotic activation of K-Cl cotransport, 450 mOsm was sufficiently high to preclude any such activation.

Hereafter, the difference in K^+ flux in the presence and absence of bumetanide (with ouabain always present) is referred to as "Na-K-Cl cotransport", and difference in K^+ flux in the presence and absence of hypertonicity (450 mOsm, with both bumetanide and ouabain always present) is referred to as "K-Cl cotransport". The K^+ flux remaining in hypertonic medium in the presence of bumetanide and ouabain is referred to as "residual" K^+ influx or efflux.

Analytical methods Concentrations of Na^+ and K^+ were measured by flame emission photometry. Total cell Mg was determined by flame absorption spectrophotometry. Osmotic concentrations of media were verified by vapor pressure osmometry. Cell ATP was determined by the method of luciferin/

luciferase assay (Brown, 1982) as previously described (Marjanovic & Willis, 1992). Cell pH was determined directly on lysates of cells as described previously (Zhao & Willis, 1993). Cell chloride was determined using a chloridometer as described previously (Zhao & Willis, 1993). Correction for chloride trapped in extracellular space in the red cell pellet was based on introducing ^{86}Rb to a cell suspension and then immediately centrifuging the suspension (15 sec, 13,000 g) on a layer of dibutyl phthalate that separates the cells from the medium. The pellets were lysed in 5 per cent trichloroacetic acid and the radioactivity determined to obtain the volume of distribution of the Rb. Cell water content was determined on centrifuged pellets of red cells weighed wet in tared beakers with a microgram balance. The samples were then dried for a minimum of 1.5 h at 90°C and 0.5 atm in a vacuum oven. (Preliminary trials showed these conditions to be sufficient to achieve full desiccation.) The samples were reweighed after drying and the water content was computed from the weight change.

Studies of acid-activated, amiloride-sensitive Na^{+} influx

Adjustment of cytoplasmic pH. The intracellular pH was set to desired levels by equilibrating cells at a 10 per cent suspension in a medium with appropriately adjusted pH. For purposes of acidification of the cells this medium contained (mM): 75 NaCl, 75 KCl, 1.5 MgCl_2 , 10 glucose and 10 mM MOPS with pH adjusted to 6.0. To prevent swelling caused by acidification (Escobales and Canessa 1986), this acid equilibrating medium was also concentrated to 360 mOsm by addition of N-methyl-D-glucamine (NMDG). Alkaline equilibration medium contained 50 mM NaCl (to yield a net osmolarity of 280 mOsm for prevention of shrinkage), 75 mM KCl, 1.5 mM MgCl_2 , 10 mM glucose and 10 mM Tris buffer at pH 8.5. To prevent hemolysis 0.1 per cent albumin was added to both media.

After 20 min equilibration at 37°C , DIDS was introduced to a final concentration of 0.125 mM and the cells were equilibrated for another 20

min. Prior to incubation the suspension was centrifuged at 13000 g for 15 seconds, the equilibration medium removed and the cells were suspended in the appropriate incubation medium with pH 7.4 for measurement of Na^+ influx (see below).

Cytoplasmic pH was measured as the pH of a lysate using a Fisher Analytical pH meter. Cytoplasmic/lysate pH was determined on a sample of cells taken before incubation and another taken after incubation. The mean of the two was used as the value reported in the data. Initial lysates were prepared by centrifuging the equilibration suspension, removing the supernatant and lysing with 0.6 ml distilled water (pH 7.0). Terminal lysates were prepared in the same way from cells suspended in the incubation medium.

Media used for studies of Na^+ influx. In early experiments (e.g., Fig. 8), the incubation medium was the same as that used to prepare the suspension of cells (see above) except that 0.1 mM ouabain was added during Na^+ influx determinations, as noted below. However, in later experiments, acidified cells were routinely incubated in a hypotonic medium with only 125 mM NaCl (260 mOsm) and mildly alkalinized cells were incubated in the same medium made hypertonic by the addition of NMDG (530 mOsm). Osmolarity of media was verified by vapor pressure osmometry. Other minor modifications and variations are noted in the text and figure legends.

Measurement of Na^+ influx. Radioactive $^{22}\text{NaCl}$, specific activity 100-1000 mCi/mg Na, was purchased from Amersham Corporation and diluted so that 20 μl contained about 300,000 cpm. Unidirectional Na influx was determined by the measurement of initial rate of ^{22}Na into cells. The 10 per cent red cell suspension (0.3 ml) was placed in 1.5 ml Eppendorf plastic microcentrifuge tubes containing ^{22}Na in the incubation medium to give a final volume of 1 ml of 3 per cent suspension (i.e., about 30 μl cells). This suspension of red cells in influx medium in 1.5 ml tubes was incubated

at 37°C or other temperatures of choice in water baths. After 5 min and after 10-15 min, triplicate samples were removed, centrifuged at 13,000g for 15 seconds and the supernatant removed by suction. The cell pellet was then washed 3 times with ice-cold wash medium (107 mM MgCl₂, 10 mM Tris, pH 7.8). The pellets were treated with 5 per cent trichloroacetic acid to precipitate protein and were spun for 1 min, then the radioactivity of the supernatant was counted in scintillation cocktail in a liquid scintillation counter.

All determinations of Na⁺ influx were done with 0.1 mM ouabain and, except for dose-response experiments, with or without 2 mM amiloride in the influx medium.

The rate of Na influx (mmol/l cell/hr) was calculated from the difference of Na entry at 5 min and 15-20 min, by using the specific activity of ²²Na and the cell volume as described abovebelow.

Measurement of H⁺ efflux. Loss of H⁺ from cells was estimated by a method described previously (Zhao & Willis 1993). Briefly, 5-10 ml of 10 per cent suspension of cells in a medium lightly buffered with 0.2 mM MOPS was placed in a chamber with a stirrer, pH electrode and inlet for solution. Initially, external pH was adjusted to be close to that of the cytoplasm. In one set of experiments with acidified cells (Fig. 14 A), pH was raised to 7.4 to initiate measurement. Later, the osmolarity of the suspension was decreased by injecting water. In another set with alkalized cells (Fig. 14 B), measurement was initiated by making the suspension hypertonic by injecting sucrose solution. In either case, the pH of the medium was held constant by titration with KOH (10 mM for unacidified cells, 75 mM for acidified cells). The volume of KOH titrant and time were recorded manually.

RESULTS

PART I. THERMAL ACTIVATION OF K-Cl COTRANSPORT PRECLUDES CELL SWELLING DURING HYPERTHERMIA

K^+ influx. As a basis for comparison, the results of Fig. 7 of the Midterm Report, describing the effect of temperature on ouabain-sensitive K^+ influx, are recast for this report in Fig. 1. Ouabain-insensitive K^+ influx, Na-K-Cl cotransport, K-Cl cotransport and residual K^+ influx as defined in Methods and Materials were measured at temperatures between 27°C and 45°C. The results (Fig. 1) showed that total passive, ouabain-insensitive K^+ influx rose steeply at temperatures above 37°C. The only measured component pathway of passive K^+ influx that exhibited a behavior parallel with that of total ouabain-insensitive K^+ influx was K-Cl cotransport (Fig. 1 A); Na-K-Cl cotransport declined gradually over the same range and residual influx rose only slightly (Fig. 1 B) with a Q_{10} of about 1.4 over the full range of temperature. It was shown in the Midterm Report that, as expected for K-Cl cotransport, activation by warming was entirely dependent upon the presence of chloride ion (Fig. 10 B of Midterm Report); there was no thermal or swelling activation in media with nitrate replacing chloride.

The increase in K-Cl cotransport caused by warming from 37°C to 41°C was of about the same magnitude as that caused by incubation of cells in hypotonic medium (210 mOsm) at 37°C (Fig. 2). Swelling activation of K-Cl cotransport is dependent upon dephosphorylation of the carrier or of some regulator thereof (reviewed by Hoffmann and Dunham, 1995), and, accordingly, is inhibited by inhibitors of phosphatases such as okadaic acid and calyculin (Jennings & Schulz, 1991; Kaji & Tsukitani, 1991; Starke & Jennings, 1993). K-Cl cotransport in guinea pig red cells was only weakly inhibited by okadaic acid at 37°C (data not shown). Calyculin (25 μ M), however, caused 93 per cent inhibition of volume-sensitive K^+ influx at

37°C (i.e., difference between influx in hypotonic and hypertonic medium), and it blocked all of the rise in K^+ influx in isotonic medium caused by warming from 37°C to 41°C (Fig. 2). In the presence of calyculin, K^+ influx in hypotonic medium was the same as in isotonic medium at all temperatures investigated, but at 41°C and 45°C influx in hypertonic medium, with or without calyculin, was less than in isotonic medium with calyculin at those temperatures. Thus, for example, while calyculin blocked about 65 per cent of isotonic K-Cl cotransport at 41°C, there was a remaining fraction of 35 per cent that was unaffected. (In one experiment raising calyculin concentration to 100 μ M had no greater effect.)

Thermal activation of K-Cl cotransport up to 45°C was fully reversible. When cells were preincubated at 41°C and then returned to 37°C for 10 minutes before flux determination, their influxes in isotonic and hypertonic media with ouabain and bumetanide were no different from those of cells preincubated and then incubated at 37°C (mean difference in hypertonically inhibitable component of 0.01 ± 0.01 for 3 experiments). Conversely, influxes at 41°C were not different in cells preincubated in 37°C compared with those preincubated at 41°C. The time-course for change in rate of uptake of isotope with a step change of temperature is shown in Fig. 3 for cells incubated in isotonic medium with ouabain and bumetanide. Instantly warming cells from 37°C to 41°C caused an apparently immediate increase in rate of uptake to that of cells incubated in 41°C continuously (Fig. 3 A, Table 1). When cells were instantly cooled from 41°C to 37°C, however, transition to the steady-state rate for 37°C occurred only after a delay of about 7 minutes (Fig. 3 B, Table 1).

The steep rise in K-Cl cotransport with warming (Fig. 1 A) corresponds to a Q_{10} of 9.7 between 37°C and 41°C and an overall Q_{10} of 5.4 between 27°C and 45°C. The foregoing results seemed to indicate that such large values of Q_{10} represented an effect of temperature on the regulation of the K-Cl cotransporter rather than a direct effect of temperature on the

transporter itself. In order to estimate the direct effect of temperature on K-Cl cotransport, independently of any modulation by regulatory factors, we sought a method for achieving a high level of activation of the carrier at each temperature. Among the methods that we tried, we achieved greatest activation at lower temperatures with a combination of hydroxylamine and hypotonicity. Using these conditions at several temperatures yielded a Q_{10} of K^+ influx of 2.6, both between 37°C and 41°C and between 32°C and 45°C (Fig. 4). This influx in hypotonic medium with hydroxylamine, of course, included any residual leak, but computing K-Cl cotransport, by subtracting influx either in hypertonic medium or in hypertonic medium with hydroxylamine, still yielded Q_{10} 's of only 2-3 (Fig. 4).

Several cytoplasmic factors can influence the activity of the K-Cl cotransporter, such as cell Mg^{2+} concentration, ATP concentration, cytoplasmic pH, chloride concentration and water content. Each of these factors was determined in red cells after incubation over a period of 30 minutes (corresponding to the ion flux determinations) and 2 hours. As shown in Table 2, none was observed to vary with temperature between 37°C and 45°C .

Unidirectional K^+ efflux. From the standpoint of its physiological role, K^+ efflux through the K-Cl cotransporter is the more relevant concern, even though K^+ influx is more accessible to precise measurement. As described in Methods, a technique was devised to measure K^+ efflux without use of radioisotopes, thus avoiding the long and potentially altering process of loading cells with radioisotope. This method consisted of measuring loss of K^+ into a K-free medium with RbCl replacing KCl. Assuming that the cell handles Rb^+ in the same way that it does K^+ , this amounts to using K^{39} as a cold isotope of K^+ .

The effect of warming above 37°C on unidirectional K^+ efflux, thus measured, was similar to that for influx, in that the K-Cl cotransport component accounted for most of the rise (Fig. 5 A). The pattern for efflux

differed from that for influx in that the rise in residual efflux was somewhat steeper with warming (Q_{10} of 2 between 37°C and 45°C; Fig. 5 B) than was the case for residual K^+ influx (Fig. 1 B); it also differed in that there was no detectable K-Cl cotransport under isotonic conditions at 37°C. No detectable K^+ efflux was observed as Na-K-Cl cotransport at 37°C, 41°C, or 45°C (Fig. 5B). Volume-activated efflux through the K-Cl cotransport pathway (i.e. difference in efflux in hypertonic and hypotonic media with bumetanide and ouabain) was large at 37°C and higher temperatures, but, given a rather large variation, virtually insensitive to temperature between 37°C (5.5 ± 1.5) and 41°C (7.5 ± 1.6) or 45°C (6.8 ± 1.7) ($P > 0.05$ for all pairs).

Balance of K^+ influxes and effluxes at elevated temperature

Having now measured the effects of temperature on the unidirectional influx and efflux of K^+ through the K-Cl cotransporter, the question remained of what the impact of activation of K-Cl cotransport was on balance of one-way K^+ fluxes and maintenance of cell volume. We approached this in several ways: Is balance of total influx of K^+ (i.e., passive and active) with total passive K^+ efflux maintained constant over a range of temperature, thus ensuring a steady-state of $[K^+]_{\text{cell}}$? If K-Cl cotransport is selectively removed, does this lead to a net inward influx of K^+ and to cell swelling at elevated temperature? Is the rise in pump uptake of K^+ with warming quantitatively balanced by increased net loss of K^+ through the K-Cl cotransporter?

Total K^+ influx vs. total K^+ efflux. Cells were incubated in K-free medium with 5 mM RbCl replacing KCl at temperatures between 20°C and 45°C. Efflux was measured as in Fig. 5 by sampling supernatants, and simultaneous Rb^+ uptake was determined in the pellets of the same samples by flame emission photometry. The ratio of influx to efflux (Fig. 6) was not statistically different from 1.0 between 27°C and 45°C ($P > 0.05$, i.e.,

influx did not differ from efflux).

Balance of K^+ influx and K^+ efflux in absence of K-Cl cotransport. In order to determine the impact of K-Cl cotransport on K^+ steady state at elevated temperatures, K^+ influx was measured using ^{86}Rb in 5 mM K^+ (as in Fig. 1) and this value was compared with K^+ efflux, in parallel cells treated as in Fig. 3. In these experiments (Fig. 7) the ratio of influx to efflux was close to 1 at both 37°C and 41°C in control cells. However, in hypertonic medium or in isotonic medium with 25 uM calyculin, influx exceeded efflux at 41°C (ratio of 1.7 and 2.2, respectively), but not at 37°C (Fig. 5). The possibility that the imbalance of bi-directional K^+ flux at 41°C was caused by an increased influx through the Na-K-Cl cotransporter was ruled out in separate experiments, that showed that bumetanide-sensitive influx was not increased by hypertonicity at 41°C and that calyculin caused only a small (0.4 mmole/l/h) and constant increase at each temperature.

Change in water content with warming in the presence and absence of K-Cl cotransport. The results of the previous section predicted that over time there should be swelling in cells treated with calyculin. Accordingly, cells were incubated for 2 hours with and without calyculin and the water content determined by dry weight/wet weight measurement. Table 3 shows that there was a statistically significant rise at 41°C and 45°C in water content of cells treated with calyculin compared both with untreated controls at 37°C and with their own controls ($P < 0.05$). There was no rise in water content at 37°C in cells treated with calyculin.

Net K^+ uptake through Na-K pump vs. net K^+ loss through K-Cl cotransporter. In order to determine whether the loss of K^+ through the K-Cl cotransporter at elevated temperature matched the increase of uptake of K^+ by the Na-K pump, measurements of unidirectional ouabain-sensitive K^+ influx and efflux were made simultaneously, and in parallel sets of cells unidirectional, hypertonically inhibited, ouabain-and-bumetanide-

insensitive K^+ influx and efflux were measured simultaneously. Net uptake through the pump was taken as the difference between ouabain-sensitive K^+ influx and ouabain-sensitive K^+ efflux, and net loss through the K-Cl cotransporter was taken as the difference between hypertonically inhibited, ouabain-and-bumetanide insensitive K^+ efflux and influx (Table 4). The increase in net K^+ uptake by the pump between 37°C and 41°C was 0.63 compared with increase in net loss through the K-Cl cotransporter of 0.76 (Table 4). Although these mean values were of comparable magnitude, there was no apparent correlation among the individual experiments between magnitude of pump increase with warming (range from 0.01 to 2.04) and magnitude of change in net loss through the K-Cl cotransporter with warming (range from -0.19 to 1.62).

PART II. AMILORIDE-SENSITIVE Na^+ INFLUX WITH AND WITHOUT ACIDIFICATION;
ACIDIFICATION WITH WARMING CAUSES EXPLOSIVE RISE IN Na^+ UPTAKE.

Dependence of amiloride-sensitive Na^+ influx on $[\text{Na}^+]_o$. In an earlier study of Na-H exchange in guinea pig red cells (Zhao & Willis, 1993), dependence of the carrier on extracellular $[\text{Na}^+]$ was determined by first activating cells either with hypertonic incubation (i.e., cell shrinkage) or by cooling (Zhao & Willis, 1993). In order to compare the Na-activation kinetics in cells that were acidified with those in cells that had been shrunk, cells were acidified to a cytoplasmic pH of 6.4-6.7 by equilibration in medium with pH 6.0 as described in Materials and Methods. They were then incubated in media with pH 7.4 and various concentrations of Na^+ achieved by substituting choline-chloride medium iso-osmotically with NaCl medium (Fig. 8). For comparison, a parallel series of unacidified cells was incubated in the same media made hypertonic by addition of excess choline chloride to achieve an osmotic concentration of 450 mOsm. The latter series yielded the same results as the comparable experiment

reported by Zhao & Willis (1993); a plateau in amiloride-sensitive Na^+ influx between 50 mM and 100 mM $[\text{Na}^+]_o$ and a second 'tail' of activation above 100 mM $[\text{Na}^+]_o$. In contrast, amiloride-sensitive Na^+ influx in acidified cells rose only gradually with increase in extracellular $[\text{Na}^+]$ up to 100 mM, but rose steeply to a value at 150 mM $[\text{Na}^+]_o$ of 140 mmoles/l cells/h, 20 fold greater than the plateau value (7 mmoles/l cells/h) in unacidified cells (Fig. 8).

Interaction of acid-activation and shrinkage-activation of amiloride-sensitive Na^+ influx. The results in Fig. 8 indicated that the two main methods of activation of amiloride-sensitive Na^+ influx - cell shrinkage and cytoplasmic acidification - yielded vastly different responses to extracellular Na^+ . If these represented a single mechanism operating in different modes, one might expect the effects of the activating procedures (cytoplasmic acidification, cell shrinkage) to be complementary. To examine this point, two cross-over experiments were done - that is, determination of effect of osmolarity on amiloride-sensitive Na^+ influx in acidified cells and effect of cytoplasmic pH in hypertonically incubated cells. In acidified cells (incubated at 45°C to enhance amiloride-sensitive Na^+ influx at low $[\text{Na}^+]_o$), decreasing osmolarity below 300 mOsm caused an increase in amiloride-sensitive Na^+ influx and raising osmolarity decreased it (Fig. 9). Conversely, in cells with pH set to various levels between pH 6.2 and 8.2 the optimum pH for amiloride-sensitive Na^+ influx was at pH 7.1 at 37°C in cells incubated in hypertonic medium (Fig. 10); modest acidification inhibited it. Thus, not only were the two types of activation not complementary, they appeared to interfere with one another.

To characterize these two different modes of activated Na^+ influx further and to avoid overlap between them, cells activated by cytoplasmic acidification in subsequent experiments were incubated in slightly hypotonic medium (260 mOsm, 125 mM NaCl), whereas cells incubated in hypertonic medium (also containing 125 mM-Na, but with N-methyl-D-glucamine

(NMDG) added to yield a minimum osmolarity of 530 mOsm) were first alkalinized to cytoplasmic pH above 7. Amiloride-sensitive Na^+ influx in alkalinized cells that were hypertonically incubated we hereafter refer to as "Type 1", and amiloride-sensitive Na^+ influx in acidified cells hypotonically incubated we refer to as "Type 2".

Effect of temperature on Type 1 and Type 2 amiloride-sensitive Na^+ influx. Preliminary evidence (Fig. 5 of Midterm Report) suggested that above 37°C warming decreased the capacity for shrinkage-activated amiloride-sensitive Na^+ influx. To confirm this observation in alkalinized cells hypertonically incubated (Type 1) and to compare it with the thermal behavior of Type 2 amiloride-sensitive Na^+ influx, cells were incubated at several temperatures between 32°C and 45°C (Fig. 11). Because 125 mM Na^+ is the highest concentration used in these experiments (to allow for hypotonicity for the acidified cells), the rate of Type 2 influx at 37°C was lower than shown in the previous experiments. Nevertheless, Type 2 amiloride-sensitive Na^+ influx rose steeply with warming. In contrast, Type 1 amiloride-sensitive influx was maximal at 37°C , and it declined at both higher and lower temperatures.

Dose-inhibition of Type 1 and Type 2 amiloride-sensitive Na^+ influx by amiloride and its analogs. At 37°C Type 1 Na^+ influx was fully inhibited by 0.2 mM amiloride, but equivalent inhibition of Type 2 Na^+ influx required 2 mM amiloride (Fig. 12). At 37°C the effects of dimethylamiloride (DMA) were similar to those of amiloride on both types of Na^+ influx except that the concentrations required were one quarter those required for amiloride (full inhibition at 50 μM for Type 1, and at 500 μM for Type 2 (Fig. 13 A). The amiloride analog, EIPA, is supposedly more selective for some Na-H exchangers than amiloride, as well as more potent. In one experiment it fully inhibited Type 1 Na^+ influx at 37°C at a concentration 10 nM, but it had no inhibitory effect on Type 2 Na^+ influx up to a concentration of 3 μM

(data not shown).

Raising the temperature from 37°C to 41°C (Fig. 10 B) caused no change in sensitivity of Type 1 Na⁺ influx to DMA (full inhibition at 50 µM), but it did result in a slight decrease in sensitivity of Type 2 (full inhibition at 1 mM). More perplexing was the observation that at 41°C lower concentrations of DMA (between 2 - 300 µM) increased Na⁺ influx in acidified cells hypotonically incubated (Fig. 13 B).

Effect of cytoplasmic Mg²⁺ on Type 1 and Type 2 amiloride-sensitive influx. The amiloride-sensitive Na-H exchanger has been reported to be dependent upon cytoplasmic free Mg²⁺ (Parker et al., 1989). To determine the dependence of the two modes of activation of amiloride-sensitive Na⁺ influx in guinea pig red cells on cytoplasmic Mg²⁺, cells were depleted of Mg²⁺ by incubation with A23187 in medium containing EDTA, a procedure previously found to lower Mg²⁺ to nonmeasurable levels (Xu & Willis, 1994). The results (Table 5) showed that Type 1 amiloride-sensitive Na⁺ influx essentially vanished in the Mg-depleted cells, whereas Type 2 amiloride-sensitive Na⁺ influx doubled in Mg-depleted cells.

H⁺ efflux coupled to Type 1 and Type 2 amiloride-sensitive Na⁺ efflux. The previous study of Na-H exchange in guinea pig red cells (Zhao & Willis, 1993) had demonstrated a Na-dependent loss of H⁺ at 37°C from cells either incubated in hypertonic medium or acid loaded and incubated in isosmotic medium, similar to that observed in human red cells (Escobales & Canessa 1986). However, the extraordinary magnitude of amiloride-sensitive Na⁺ influx in acidified cells observed in this study in hypo-osmotic medium, especially at 41°C, raised anew the question of whether this represented an exchange for cytoplasmic H⁺. To answer this question, acidified cells were placed in a non-buffered medium and pH of the medium was held constant by titration of KOH. The time-course of titration (Fig. 14 A) showed that there was no change in rate of loss of H⁺ after the addition of water to the medium to make it hypo-osmotic, nor was there a difference in the rate of

medium to make it hypo-osmotic, nor was there a difference in the rate of H^+ loss between $37^{\circ}C$ and $41^{\circ}C$ after the addition of water. To verify the adequacy of the method to exhibit a change in rate of loss if one were present, H^+ loss was measured in a suspension of alkalized cells to which solute was added to create hypertonicity (Fig. 14 B) and a marked rise in H^+ loss was observed.

Net gain of Na^+ compared with unidirectional Type 2 amiloride-sensitive Na^+ influx. The previous result seemed to indicate that no H^+ extrusion could be specifically identified with the large amiloride-sensitive Na^+ influx of Type 2 especially at elevated temperature. Another possibility was that those large values represented in whole or in part an accelerated exchange of extracellular Na^+ for intracellular Na^+ . To test this possibility, parallel sets of cells were incubated in the same manner with and without ^{22}Na . After incubation, entry of isotopic Na^+ was determined as before and in the nonradioactive cells Na^+ content was determined by flame photometry (Table 6). Net uptake of Na^+ , measured by flame photometry, matched one-way Na^+ entry, measured isotopically, at $37^{\circ}C$ and accounted for well over half of amiloride-sensitive Na^+ influx at $45^{\circ}C$. Thus, Na-for-Na exchange need not be postulated for $37^{\circ}C$, and while its possibility at $45^{\circ}C$ cannot be excluded, it would not have accounted for most the isotopic influx observed at that temperature.

Effect of calyculin on shrinkage-activated Na^+ influx at elevated temperature. In the Midterm Report, the hypothesis was proposed that rise in K-Cl cotransport and decline of shrinkage-activated Na-H exchange with warming was a case of coordinate control. The evidence presented in Part I, above, seemed to implicate a rise in phosphatase activity as the likely determinant of increased K-Cl cotransport at elevated temperature. If that is the case and if Na-H exchange (or 'Type 1' AS Na^+ influx) is coordinately regulated, then inhibition of phosphatase ought to prevent the decline in AS Na^+ influx in hypertonically incubated cells. To test this

possibility, cells were incubated in hypertonic medium with and without calyculin at temperatures between 37°C and 45°C (Fig. 15). The results showed that while there was a slight elevation of amiloride-sensitive Na⁺ influx at 37°C, there was no significant effect at higher temperatures.

DISCUSSION

Overview

This project set out to determine whether there were any features of membrane transport of Na^+ and K^+ in a model system, the guinea pig red cell, that might offer insight into performance and failure of cells at work in a hyperthermic human subject. In the process two relevant and wholly unsuspected phenomena have been discovered: (1) thermal activation of K-Cl cotransport, that may at the same time protect cells from swelling at elevated temperatures and increase the risk of hyperkalemia and (2) enormously amplified amiloride-sensitive Na^+ influx in acidified cells at elevated temperature, that may lend credibility to one aspect of the "Energy Depletion Hypothesis". We ran out of time before we could evaluate the impact of the second of these discoveries on the cell's Na-K pump (and through it, metabolism) and on cell volume regulation. We have also not been able to explore these features in alternative cell types (as proposed in a supplemental request) nor in human red cells *in vitro* and (in collaboration with USARIEM) *in vivo* (as originally envisioned as a long range goal of this project). Even so, the guinea pig red cells have provided a useful beginning to the understanding of these two phenomena, and both are discussed below.

The Thermal Activation of the K-Cl Cotransporter

The results of Part I (Fig. 1, Fig. 3) demonstrate that raising temperature does not 'just make cell membranes leaky' to K^+ . On the contrary, what we might call true "leakiness" (residual K^+ flux) rises only gradually with warming, with a Q_{10} about 2 for efflux and about 1.4 for influx. One component, Na-K-Cl cotransport, actually declines with warming, but in any case does not appear to contribute to net K^+ loss at temperatures between 37°C and 45°C (Figs. 1B and 2B). There is, however, a steep rise of unidirectional K^+ influx and efflux at temperatures above 37°C , and this is mostly due to the increase of K-Cl cotransport (Figs. 1

- 3), which results in net loss through this pathway at elevated temperatures (Table 4).

Several investigators have noted a large temperature dependence of the activation of the K-Cl cotransport carrier by swelling in red cells of human, sheep and rabbit over the range of 37°C to 25°C (Lauf, 1983; Ellory, Hall and Stewart, 1985; Jennings & Al-Rohil, 1990). Those findings were extended by Jennings & Al-Rohil (1990), who showed that the thermal dependence of K-Cl cotransport was quite small (Q_{10} of about 1.5 between 25°C and 37°C) in rabbit red cells that were already strongly activated by pre-exposure to N-ethyl maleimide (NEM). They interpreted this to mean that activation by swelling at low temperature (25°C) occurred more slowly and reached a lower maximum. We in turn have extended those results by demonstrating that even in isotonic medium a rise in temperature above 37°C strongly activates the system.

Mediation of thermal activation. More recently, the activation of the K-Cl cotransport system by cell swelling, though complex, has been shown to involve in part the dephosphorylation of the carrier itself or of a regulator of the carrier (for reviews see Lauf, Bauer, Adragna, Fujise, Zade-Oppen, Ryu & Delpire, 1992; Lauf, Adragna & Agar, 1995; Hoffmann & Dunham, 1995). The large thermal activation observed in the present study in the absence of cell swelling appears to depend upon alteration in the balance of these regulatory pathways, as distinct from a direct effect of temperature on the carrier itself. This proposition rests upon three of our observations: (1) The low temperature dependence of K-Cl cotransport when already highly activated by swelling and hydroxylamine (Fig. 4); (2) the inhibitory effect of the phosphatase inhibitor, calyculin (Fig. 2); and (3) the delayed effect of lowering temperature from 41°C to 37°C (Fig 3 B). We will consider each of these points.

When, like Jennings and Al-Rohil (1990), we used NEM initially to activate K-Cl cotransport to obtain a Q_{10} at temperatures above 37°C in guinea pig red cells, we found that K^+ influx in NEM-treated red cells actually declined with warming (Willis & Anderson, 1995). This result is well explained by the findings of Lauf and Adragna (1995) of thermally activated inhibitory NEM binding sites. Accordingly, we sought other means for achieving high activation throughout the temperature range used in this study. Lauf (1990) had observed an extraordinary level of activation by hydroxylamine in LK sheep red cells at 37°C (greater than with NEM) and no further increase in hypotonic medium with that activator. In guinea pig red cells we found in trial experiments that the combination of hydroxylamine and hypotonicity gave a higher K^+ influx at temperatures below 37°C than either agent alone.

K^+ influx in hydroxylamine and hypotonic medium yields a Q_{10} of 2.6 over the relevant ranges (Fig. 4). This influx includes both highly activated K-Cl cotransport and any residual leak. Since the hydroxylamine may make the cells more leaky to K^+ , it is problematic how much of this large K^+ influx should be subtracted out as baseline (i.e., K^+ influx in hypertonic medium, whether with or without hydroxylamine, see Fig. 4). Under any assumption, however, it is clear that the relative increase with warming of the already highly activated carrier is far less than that of untreated cells isotonically incubated. This leads to the same conclusion as that of Jennings and Al-Rohil (1990), vis-a-vis the effects of cooling, i.e. the large effect of temperature on K-Cl cotransport operates through regulation of transport rather than directly through the transporter itself. This conclusion, based on K^+ influx, is also supported by the low Q_{10} 's for already swelling activated K^+ efflux above 37°C (2.2 for 37°C-41°C and 1.3 for 37°C-45°C).

If thermal activation involves inhibition of dephosphorylation, then it should be blocked by inhibitors of phosphatases. Both calyculin A and

okadaic acid have been widely used for this purpose in studies of activation of K-Cl cotransport by swelling (Jennings & Schulz, 1990; Kaji & Tsukitani, 1991; Starke & Jennings, 1993). In guinea pig red cells, we found okadaic acid to be largely ineffective as an inhibitor of either volume-activated or thermally activated K^+ influx, perhaps because of poor permeation of the inhibitor to the site of action (Namboodiripad & Jennings, 1996). At 37°C in guinea pig red cells calyculin was as effective in preventing swelling activation of K^+ influx as it is at that temperature in rabbit red cells. It also prevented all of the increase due to warming at 41°C and to this extent bears out the expectation (Fig. 2).

However, 25 to 30 per cent of the total volume-responsive K^+ influx (i.e. difference between hypotonic and hypertonic media, both containing ouabain and bumetanide) remained in the presence of calyculin at 41°C and 45°C (Fig. 2). This calyculin-insensitive component that rises with warming and is inhibited in hypertonic medium may represent one or a combination of several possibilities: (1) nonenzymatic (nonphosphatase) mediated dephosphorylation; (2) ordinary (i.e., direct) thermal activation of a fraction of a dephosphorylated pool of transporters not blocked by calyculin at 37°C; (3) thermal effect on regulatory factors of K-Cl cotransport that are independent of phosphorylation (e.g. second-stage increase of affinity of K-Cl cotransporter for K^+ , such as reported by Dunham, Klimczak & Logue, 1993, for swelling activation).

That the large temperature effect between 37°C and 41°C cannot be attributed mainly to a direct kinetic effect on the transporter itself is demonstrated by the 7 minute lag that it took for the rate of influx to 'relax' to the rate characteristic of 37°C after cooling from 41°C (Fig. 3 B, Table 1). It should be recognized, however, that the rates illustrated in Fig. 3 and Table 1 necessarily included residual K^+ influx. For the sake of this discussion we have assumed that rate of flux through this pathway

changes instantly with change in temperature in either direction. Because flux through this pathway is very small and because the change in this flux between 37°C and 41°C is even smaller, we were unable to test this assumption directly.

The present results with thermal activation imply that there is a greater effect of warming on activity of a phosphatase proximal to the carrier than on activity of an equivalently proximal inactivating kinase. By this interpretation, the slower effect of cooling from 41°C to 37°C (Fig. 3 B) would be attributable to a slow depletion of activated carrier by a relatively unchanged kinase reaction.

Physiological significance. Two possible consequences of the activation of net loss of K^+ through the K-Cl cotransport pathway at elevated temperature were identified at the opening of this Discussion - hyperkalemia and prevention of cell swelling. Hyperkalemia is a common but variable feature of passive hyperthermia in intact mammals (reviewed by Francesconi, Willis, Gaffin & Hubbard, 1997). Whether or not hyperkalemia will occur in hyperthermia will depend on the effect of temperature on the Na-K pump. If pump rate is increased, then increased loss of K^+ may balance that increase. If on the other hand, the pump begins to fail at elevated temperature, then increased K^+ loss from the cell will result in hyperkalemia. The results shown in Figs. 4 and 5 and Table 4 suggest under the idealized conditions of *in vitro* incubation, on average, net K^+ loss was about the same as net increase through the pump. However, *in vivo* and over longer intervals, the factors that might lead to insufficient pump activity (reduced ATP delivery, lowered cell pH) are the very ones that could accentuate the loss of K^+ through the K-Cl cotransporter. Thus, whether the pump is stimulated or failing at high temperature, swelling of the cells would be predicted, and K-Cl cotransport would be a defense against that swelling. But, in the case of failing pumps, that defense would be at the expense of hyperkalemia.

Anomalous, heat-acid-swelling activated, amiloride-sensitive Na^+ influx

Part II of the Results exhibits two patterns of amiloride-sensitive Na^+ influx, markedly different in their kinetics of activation by transported ligands, Na^+ and H^+ , different in their sensitivity to inhibitors, different in their capacity for transport, opposite in their activation by change in cell volume and by change in cytoplasmic Mg^{2+} , and opposite in their response to warming at temperatures above 37°C . These differences (summarized in Table 7), raise the question of whether they represent two entirely separate and distinct pathways or merely a single pathway behaving in two different modes. While the extent of the differences would seem to exclude the latter, precedents exist for a putative single carrier pathway with alternative properties dependent upon mode of activation (Cala and Maldonado, 1994) and for carriers exhibiting channel-like characteristics under unusual conditions (Garcia-Romeu *et al.* 1996). Thus, while unlikely, a single pathway hypothesis cannot be ruled out entirely.

The pattern that we have characterized as "Type 1" amiloride-sensitive Na^+ influx conforms in every respect to the expectations of a conventional Na-H exchanger (most likely, NHE1). It responds to cell shrinkage, is inhibited by low concentrations of amiloride and DMA and by exquisitely low concentrations of EIPA and is dependent upon cytoplasmic Mg^{2+} .

So the question becomes, what is the nature of Type 2 amiloride-sensitive Na^+ influx? If it does not represent an altered activity of Type 1 (presumptively NHE1) activity, it must reflect the presence of another pathway. A simple possibility is that it represents an artifact of membrane rupture produced by the combination of swelling, acidification and elevated temperature. This possibility seems to be ruled out by the fact that it is inhibited by amiloride; in the absence of the inhibitor that combination of factors had little effect on Na^+ influx (cf. Table 6).

Of the known Na-H exchange isoforms, the one that most closely resembles the phenomena we report here is NHE3. Like Type 2 amiloride-sensitive Na^+ influx, NHE3 has a much lower sensitivity to amiloride and its analogs than NHE1 (Tse et al. 1993; Wakabayashi et al. 1997), and it is inhibited by hyperosmolarity (Kapus et al. 1994; Nath et al. 1996). However, NHE3 is an efficient transporter of H^+ (Kapus et al. 1994; Soleimani et al. 1994; Nath et al. 1996). Furthermore, it is inhibited by ATP depletion (Kapus et al. 1994), a property that would not accord with the increased amiloride-sensitive Na^+ influx seen in Mg-depleted cells (Table 5), and it has normal Na^+ activation kinetics (Nath et al. 1994).

In fact, the peculiar Na^+ activation kinetics (Fig. 8) and the extraordinarily high transport rates (Figs. 8 and 10) observed for Type 2 amiloride-sensitive Na^+ influx do not fit with any kind of a conventional carrier. Conceivably, these kinetics combined with the observation that net uptake of Na^+ occurs through this route beyond any exchange either for H^+ or Na^+ (Fig. 12B, Table 6), might fit the notion of a channel regulated by intracellular H^+ and by extracellular Na^+ . Stimulation of amiloride-sensitive influx by DMA, such as that described in Fig. 12 B, have also been reported for the ENaC channel of frog skin (Li & DeSousa 1979; Li & Lindemann 1983). Furthermore, amiloride-sensitive Na^+ channels have recently been shown to be activated by stretch (Awayda et al. 1995, Achard et al. 1996). Finally, mechanical activation lowers the amiloride sensitivity of ENaC channels (Awayda et al. 1995), although not to the extent observed in this study.

The low sensitivity to amiloride of Type 2 amiloride-sensitive Na^+ influx, uncharacteristic of ENaC channels (Garty & Palmer 1997), raises the question of whether the phenomenon of Type 2 influx accounts for the 'tail' observed in shrinkage-activated Na^+ influx (Fig. 8 and Zhao & Willis, 1993, Fig. 3). In the earlier studies (Zhao & Willis, 1993) the 'tail' could be

eliminated with only 5 μ M amiloride. The large influx induced under Type 2 conditions was unaffected by such a low concentration (Fig. 12).

Conceivably, the effect of acidification observed in Fig. 8 may have been an indirect consequence of cell swelling, so that the 'tail', which would correspond to only a minute fraction of contribution by the Type 2 mechanism in unacidified cells with normal volume, might have had an elevated sensitivity to amiloride in the absence of cytoplasmic acidification or cell swelling.

Physiological significance. Although Type 2 amiloride-sensitive Na^+ influx may be an anomaly, it could, if found to be a general phenomenon of mammalian cells, have serious physiological consequences. The combination of high temperature and cytoplasmic acidification are met in cases of exercise in a hot environment (Hubbard & Armstrong 1988) and might be further exacerbated by ATP depletion during exhaustion, which would lower free cytoplasmic Mg^{2+} . The Energy Depletion Hypothesis postulates that increasing Na^+ permeation with cellular warming, particularly through a Na-H exchanger, could account for excess stimulation of the Na-K pump (Hubbard et al. 1987). This in turn could have two dire consequences for the organism, increased heat production with runaway warming of the cell and exhaustion of energy reserves, both leading ultimately to heatstroke. Although the results of this study indicate that the conventional, shrinkage-activated Na-H exchanger actually shuts down with warming, the explosive rise in Na^+ influx in warmed acidified cells would, if generalized, fit the scenario of the Energy Depletion Hypothesis.

CONCLUSIONS

With regard to the four questions from the Introduction that were posed in the Conclusion of the Midterm Report:

1. Is the very large effect of warming on K-Cl cotransport operating through regulation of the transporter rather than directly on the transporter itself? Yes, clearly.

It appears very likely that the main effect of warming is mediated by a relatively larger increase in phosphatase activity than in kinase activity.

1A. Is there coordinate regulation of Na-H exchange and Na-K-Cl cotransport (i.e., is the decrease in these pathway mediated by the same process that is increasing K-Cl cotransport?). Apparently not.

The decrease in shrinkage-activated Na-H exchange with warming is not affected by phosphatase inhibition, even though this largely blocks the rise in K-Cl cotransport.

2. Is the large increase in K-Cl cotransport with warming balanced by the Na-K pump. Yes, under the conditions of our experiments.

However, in individual experiments there may be large net loss of K^+ when the pump does not increase sufficiently with temperature. The increase in K-Cl cotransport would always be a defense against cell swelling, whether or not Na-K pump activity is increasing in parallel. But under stringent conditions, it could contribute to hyperkalemia.

3. Does Na-K-Cl cotransport play a role in modulating ion balance at high temperature? No, not so far as we have been able to establish.

The Na-K-Cl cotransport does not account for any apparent net transport in these cells at or above 37°C under isotonic conditions (Presumably the observed unidirectional K^+ influx through this pathway represents a 1:1 exchange for cytoplasmic K^+).

4. Does increased acidity at higher temperatures contribute to greater leakiness of the plasma membrane for Na^+ ? We cannot say for certain that it does, but it is very clear that it can.

The explosive rise in Na^+ influx, and net Na^+ uptake, in acidified and warmed red cells is extraordinary. The degree of acidification achieved here is beyond that which cells would be expected to experience, but the magnitude of the effect is such that even a slight increase in acidity (and possibly attendant increase in cell volume) would cause a significant rise in Na^+ entry. If this phenomenon is general, then it poses a major threat to cells working in a warm environment.

Finally, it should be noted that there is a complementarity between the two major findings of this project. The large increase in Na^+ permeation in warmed, acidified cells probably requires increase in cell volume to occur. The activation of the K-Cl cotransporter is, at least in these cells, the only safeguard against that increase occurring under physiological conditions.

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Table 1. Delay in change of rate of K^+ influx after a step change in temperature.

Procedure	(n)	Identity	Slope ($\times 10^{-3}$) (mmole/l/min)	Delay (min)
Step warming	(3)	Control, 37°C	2.1 ± 0.5	-
		Control, 41°C	4.0 ± 1.3	-
		Shifted, 37°C \rightarrow 41°C	4.0 ± 1.0	0 ± 3
Step cooling	(3)	Control, 37°C	3.0 ± 0.4	-
		Control, 41°C	5.1 ± 0.7	-
		Shifted, 41°C \rightarrow 37°C	2.3 ± 0.3	7 ± 2

Slopes are computed from regression lines of all the data for controls (see Fig. 3) and of the last four data points for the temperature-shifted cells. The "delay" was calculated by subtracting the actual time of warming or cooling from the calculated time at which the regression curve for the warmed or cooled cells diverged from that of the control cells. (The "control cells" were those at the same temperature as the initial temperature of the temperature-shifted cells, i.e., 37°C control for step-warmed cells and 41°C for step-cooled cells).

Table 2. Cell water content and cell concentrations of chloride, total magnesium, ATP and hydrogen ion at elevated temperatures.

		37°C	41°C	45°C
		mmole/l cells		

[Cl ⁻] _{cell}	(n)			
<30 min	4	79.8 ± 3.3	82.2 ± 4.1	81.5 ± 5.4
2 h	4	79.4 ± 3.4	80.4 ± 3.5	79.1 ± 3.5
[Mg] _{cell}				
<30 min	4	2.33 ± 0.04	2.41 ± 0.11	2.36 ± 0.11
2 h	4	2.40 ± 0.15	2.37 ± 0.12	2.33 ± 0.18
[ATP] _{cell}				
<30 min	3	1.65 ± 0.08	1.58 ± 0.05	1.62 ± 0.02
2 h	4	1.31 ± 0.08	1.69 ± 0.06	1.59 ± 0.06
pH _{cell}				
<30 min	1	7.25	7.24	7.23
2 h	4	7.28 ± 0.03	7.25 ± 0.03	7.24 ± 0.02
Cell water		_____g H ₂ O/g dry wt._____		
<30 min	3	2.13 ± 0.05	1.99 ± 0.04	2.07 ± 0.20

Table 3. Water content of guinea pig red cells incubated 2 hours with and without calyculin (25 nM).

	gH ₂ O/g dry weight		
	37°C	41°C	45°C
Control	1.84 ± 0.03	1.91 ± 0.05	1.89 ± 0.04
+calyculin (25 nM)	1.87 ± 0.03	2.09 ± 0.08 ^{a,b}	1.99 ± 0.06 ^{a,b}

(a) P<0.05 for difference from control at 37°C

(b) P<0.05 for difference from control at same temperature (paired T-test).

N=6 in all cases.

Table 4. Comparison of net fluxes of K^+ through the Na-K pump and K-Cl cotransporter at 37°C and 41°C.

	Net gain of K^+ via Na-K pump	Net loss of K^+ via K-Cl cotransporter
	<u>mmole/l cells/h</u>	
37°C	1.35 ± 0.12	0.11 ± 0.07
41°C	1.98 ± 0.25	0.87 ± 0.18
Change due to warming	0.63 ± 0.25	0.76 ± 0.19

Results represent means of 9 experiments ± S.E. In parallel sets of cells ouabain-sensitive influx and efflux and hypertonically inhibited K^+ influx and efflux were measured simultaneously. "Net gain of K^+ via the pump" was calculated from the difference of ouabain-sensitive influx and efflux. "Net gain via K-Cl cotransporter" was calculated as the difference of hypertonically inhibited influx and efflux.

Table 5. Effect of Mg-depletion on amiloride-sensitive Na^+ influx at 41°C .

		Amiloride-sensitive Na^+ influx	
		(mmoles/l cells/h)	
	(N)	Type 1	Type 2
Control	4	15.3 ± 1.7	25.4 ± 5.0
Mg-depleted	4	0.9 ± 0.3	56.8 ± 8.5

Prior to determination of Na^+ influx cells were preincubated either in standard medium or in medium containing 3 μM A23187 and 0.3 mM EDTA at 37°C for 15 minutes. The latter effectively depletes free Mg^{2+} to near nil (Xu & Willis, 1994). They were then washed 4 times in the same medium but without A23187 and with 0.1% bovine serum albumin before being introduced to incubation medium for flux determination.

Table 6. Comparison of net change of cell $[Na^+]$ with unidirectional entry of Na^+ measured isotopically under "Type 2" conditions (i.e., acidified cells incubated hypotonically).

				(mmole/l cells)	
Condition	Time (min)	Temperature (°C)	(N)	Na ⁺ entry*	Change in $[Na^+]_{cell}$ **
Control	5	37	4	2.3 ± 0.4	3.2 ± 0.4
Amiloride	5	37	4	1.4 ± 0.1	1.4 ± 1.0
Control	20	37	4	7.6 ± 1.2	10.2 ± 1.8
Amiloride	20	37	4	1.9 ± 0.1	1.7 ± 0.6
Control	5	45	3	29.6 ± 4.7	21.5 ± 3.6
Amiloride	5	45	3	4.0 ± 0.4	5.1 ± 1.3
Control	20	45	3	50.3 ± 4.1	33.8 ± 3.4
Amiloride	20	45	3	7.1 ± 1.8	8.9 ± 2.9

*"Na entry" was the one-way entry as computed from uptake of ^{22}Na and from specific activity. Subtraction of the 5 min value from the 20 min value and multiplication by 4 would yield unidirectional influx.

** An initial cell sample was taken before onset of incubation to determine starting cell $[Na^+]$. Values shown are increments above this initial value.

Table 7. Summary of two patterns of amiloride-sensitive Na^+ influx in guinea pig red cells.

	Type 1	Type 2
Acid pH_i	Not essential	Strongly activates
Cell shrinkage	Activates	Inhibits
Cell swelling	Inhibits	Activates
Warming	Inhibits	Strongly activates
$[\text{Mg}^{2+}]_{\text{cell}} > 0$	Required	Not required
Effect of $[\text{Na}]_o$	Saturates, $< 100 \text{ mM}$	Steep rise, $> 100 \text{ mM}$
Maximum transport rate	$\sim 10 \text{ mmol/l cells/h}$	$> 100 \text{ mmol/l cells/h}$
Coupled to H^+ efflux?	Yes	No
Amiloride inhibition	0.2 mM	2 mM
$100 \text{ }\mu\text{M DMA (41}^\circ\text{C)}$	Inhibits	Stimulates

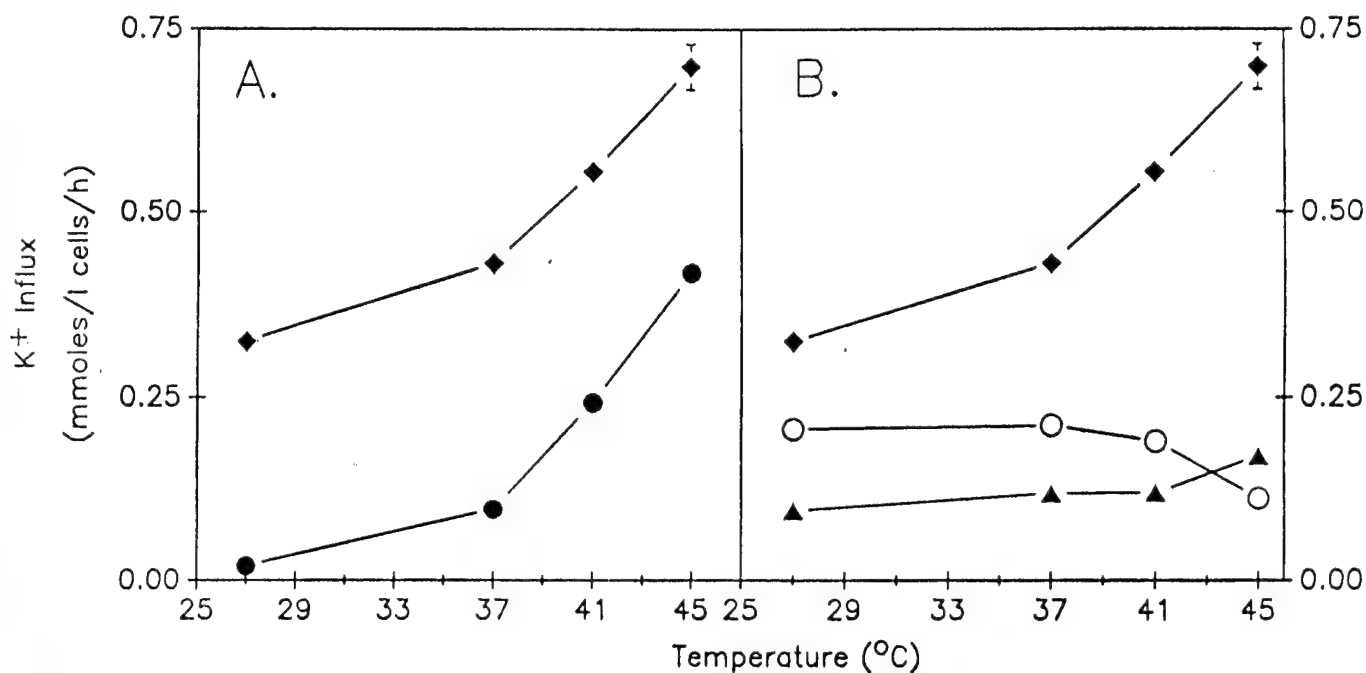


Fig. 1. Effect of temperature on components of ouabain-insensitive K⁺ influx. A. Diamonds, ouabain-insensitive influx; closed circles, K-Cl cotransport. K-Cl cotransport was computed by subtracting influx in hypertonic medium (with 10 μ M bumetanide and 100 μ M ouabain) from influx in isotonic medium containing ouabain and bumetanide. B. Diamonds, ouabain-insensitive K⁺ influx (same data as A, repeated for comparison); open circles, Na-K-Cl cotransport; closed triangles, residual K⁺ influx. Cells were incubated as described in Methods. Na-K-Cl cotransport was bumetanide-sensitive component of influx in presence of ouabain; residual influx was that remaining in hypertonic medium (450 mOsm) in the presence of ouabain and bumetanide. Results are means \pm S. E. (brackets shown where larger than symbols) of 3-6 experiments.

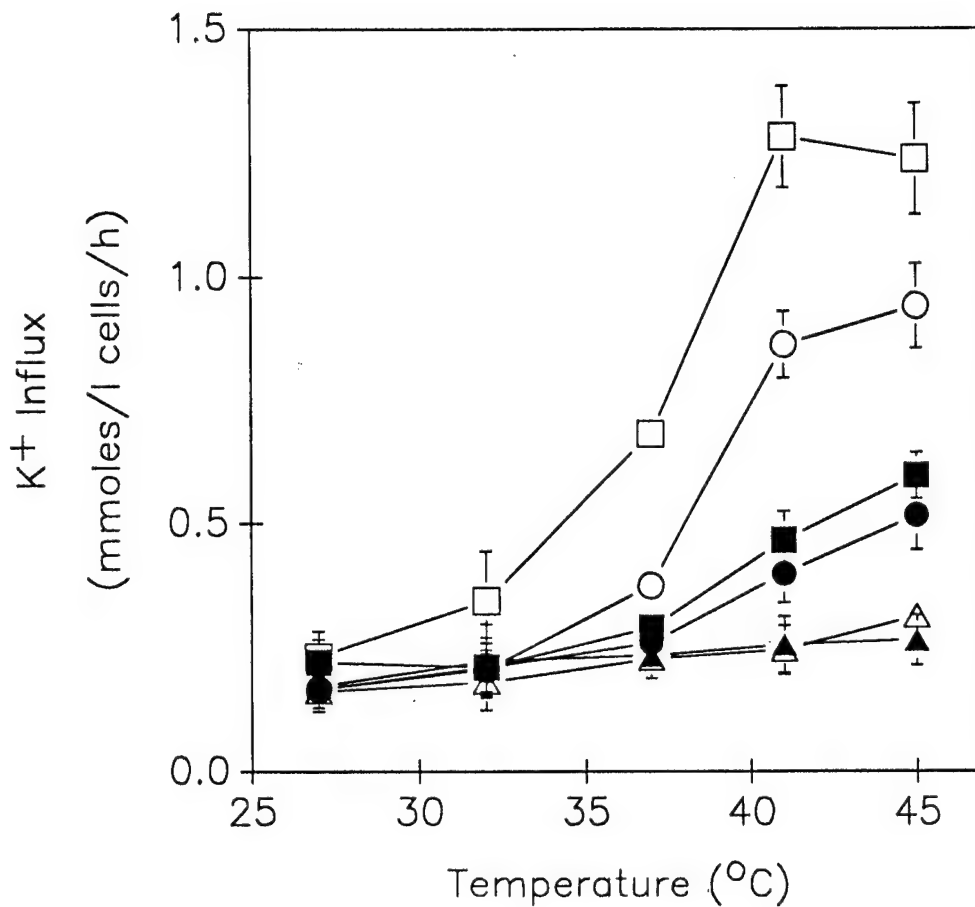


Fig. 2. Inhibition by calyculin of thermal activation and swelling activation of K-Cl cotransport. Open symbols, no calyculin; closed symbols, with 25 nM calyculin. Triangles, hypertonic; circles, isotonic; squares, hypotonic. Cells are incubated in presence of ouabain (100 μ M) and bumetanide (10 μ M). Means of 5 experiments \pm S.E. are shown.

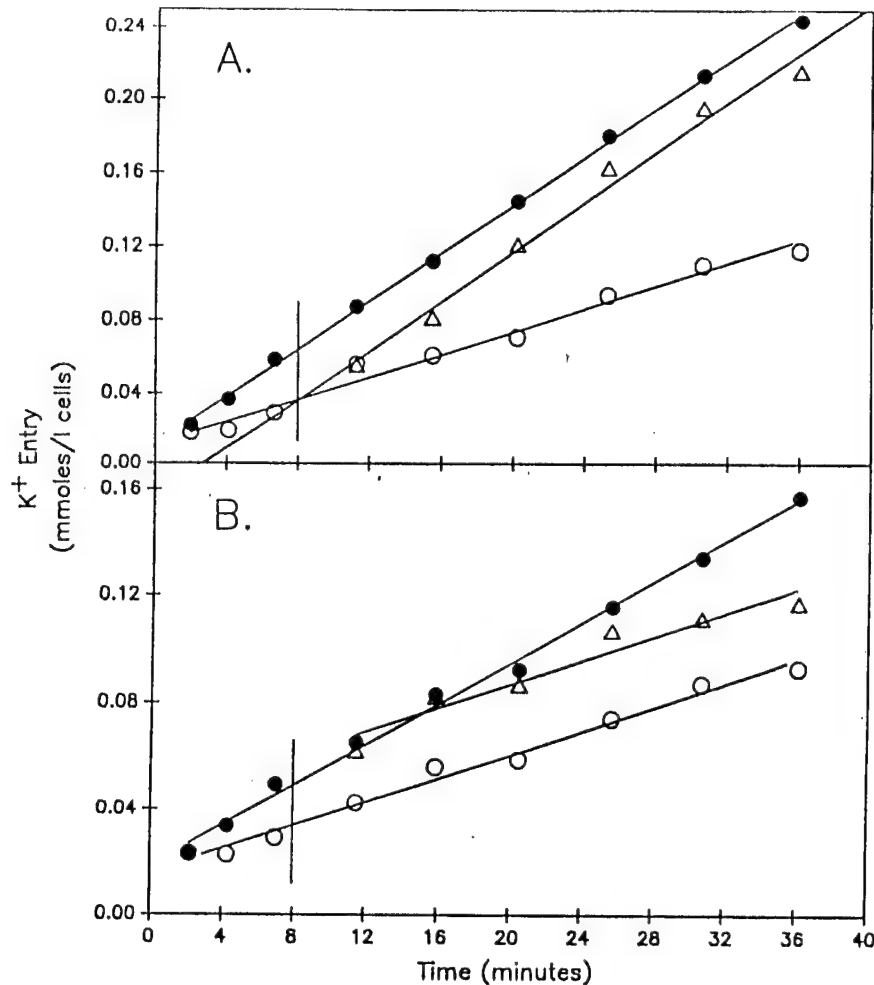


Fig. 3. Time-course of effect of rapid increase (A) or decrease (B) in temperature on ouabain-and-bumetanide insensitive K^+ influx. Cells were incubated in isotonic medium with ^{86}Rb , 100 μM ouabain and 10 μM bumetanide and samples were withdrawn at intervals as shown. At the point marked by the vertical line part of the suspension was removed, placed in a pre-equilibrated container and an equal volume of medium was added at a temperature previously determined to cause a change in temperature either from 37°C to 41°C (panel A) or from 41°C to 37°C (panel B) after rapid mixing. The remaining suspension was diluted by an equal volume of isothermal medium. The other control suspension (i.e., 41°C in panel A and 37°C in panel B) was also diluted by addition of isothermal medium. Filled circles, 41°C throughout; open circles, 37°C throughout; triangles, temperature-shifted cells (panel A, rapidly warmed from 37°C to 41°C; panel B, rapidly cooled from 41°C to 37°C). Regression lines are shown for each set of points. Results are for a single experiment of each type, representative of 3. Group data for slope of curves and delay of onset of change in slope are shown in Table 1.

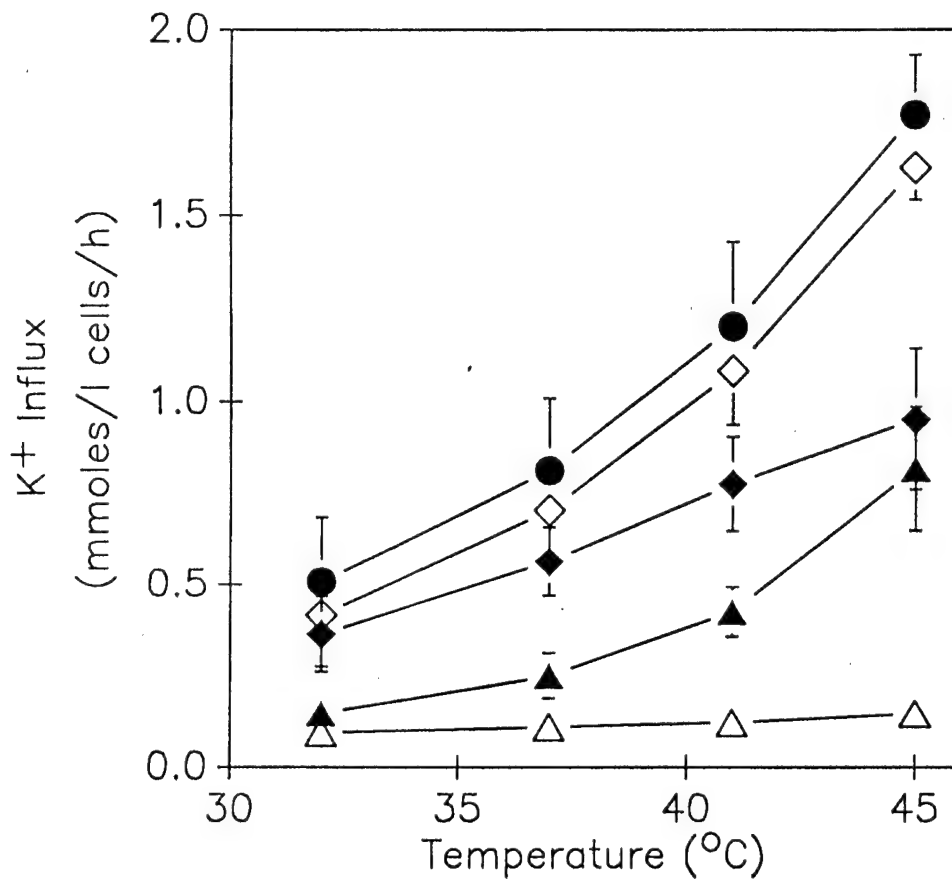


Fig. 4. Effect of temperature on K^+ influx in cells incubated in hypotonic medium with hydroxylamine. Combination of hydroxylamine and hypotonic incubation was used as a means of strongly activating influx through the K-Cl cotransporter at all temperatures. All cells were incubated with ouabain and bumetanide. Closed circles, influx in hypotonic medium (210 mOsm) with hydroxylamine (1 mM); Q_{10} is 2.6 for 32°C-45°C and for 37°C-41°C. Open diamonds, "K-Cl cotransport", calculated by subtracting residual K^+ influx, open triangles, from K^+ influx in hypotonic medium with hydroxylamine; Q_{10} is 2.9 for 32°C-45°C and for 37°C-41°C. Closed diamonds, "K-Cl cotransport", calculated by subtracting influx in red cells incubated in hypertonic medium with hydroxylamine (closed triangles) from influx in hypotonic medium with hydroxylamine; Q_{10} is 2.1 for 32°C-45°C and 1.9 for 37°C-41°C. Means \pm S.E. for 3 cases are shown.

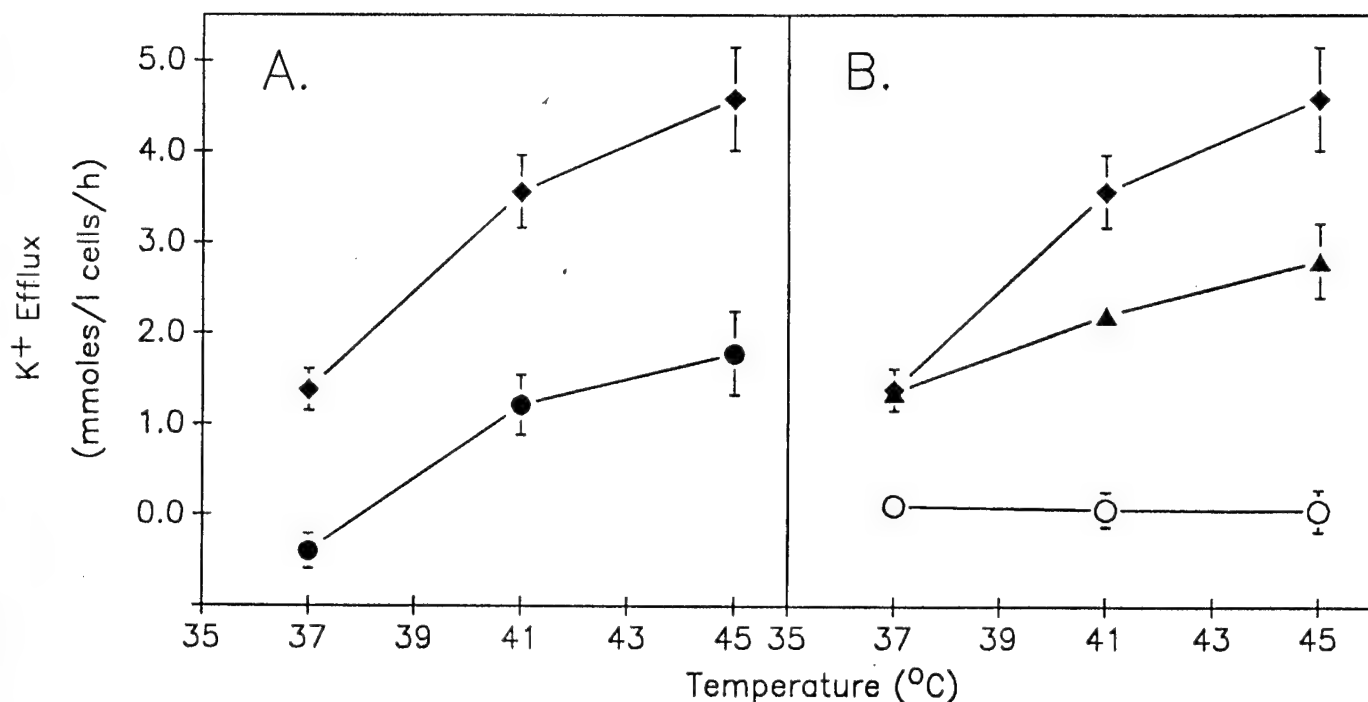


Fig. 5. Effect of temperature on unidirectional K⁺ efflux. All cells were incubated with 100 μ M ouabain. K⁺ efflux was measured as K⁺ loss into K-free medium with 5 mM RbCl substituted for KCl. Initial loss after 5 min was subtracted from loss at a later interval (20-25 min) and the difference was used to compute rate. K⁺ loss due to lysis was corrected by measurement of loss of hemoglobin or protein. Cells were incubated with 0.5 per cent albumin to minimize hemolysis. A. Diamonds, ouabain-insensitive K⁺ efflux; closed circles, K-Cl cotransport. K-Cl cotransport was computed by subtracting efflux in hypertonic medium (with bumetanide, 10 μ M and ouabain 100 μ M) from efflux in isotonic medium containing ouabain and bumetanide. B. Diamonds, ouabain-insensitive K⁺ efflux (same data as A, repeated for comparison); open circles, Na-K-Cl cotransport; closed triangles, residual K⁺ efflux. Na-K-Cl cotransport was bumetanide-sensitive component of efflux in presence of ouabain; residual efflux was that remaining in hypertonic medium (450 mOsm) in the presence of ouabain and bumetanide. Results are means \pm S. E. (error bars shown where larger than symbols) of 6 experiments.

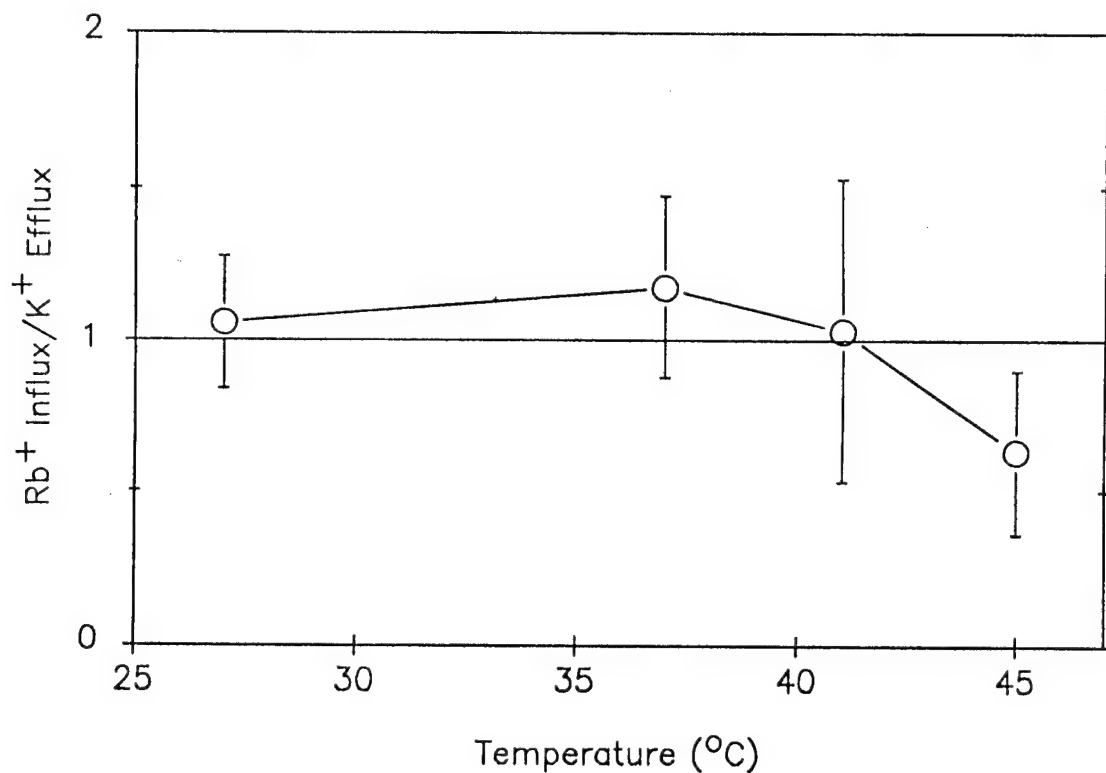


Fig. 6. Effect of temperature on balance of K^+ (Rb^+) influx and K^+ efflux. Efflux was determined as described in Fig. 6 except that no inhibitors were present. In the same samples pelleted red cells were washed three times in isotonic Tris-buffered $MgCl_2$ medium and influx determined as the uptake of Rb^+ as determined by flame emission photometry. Points represent means \pm S.E. of 5 experiments.

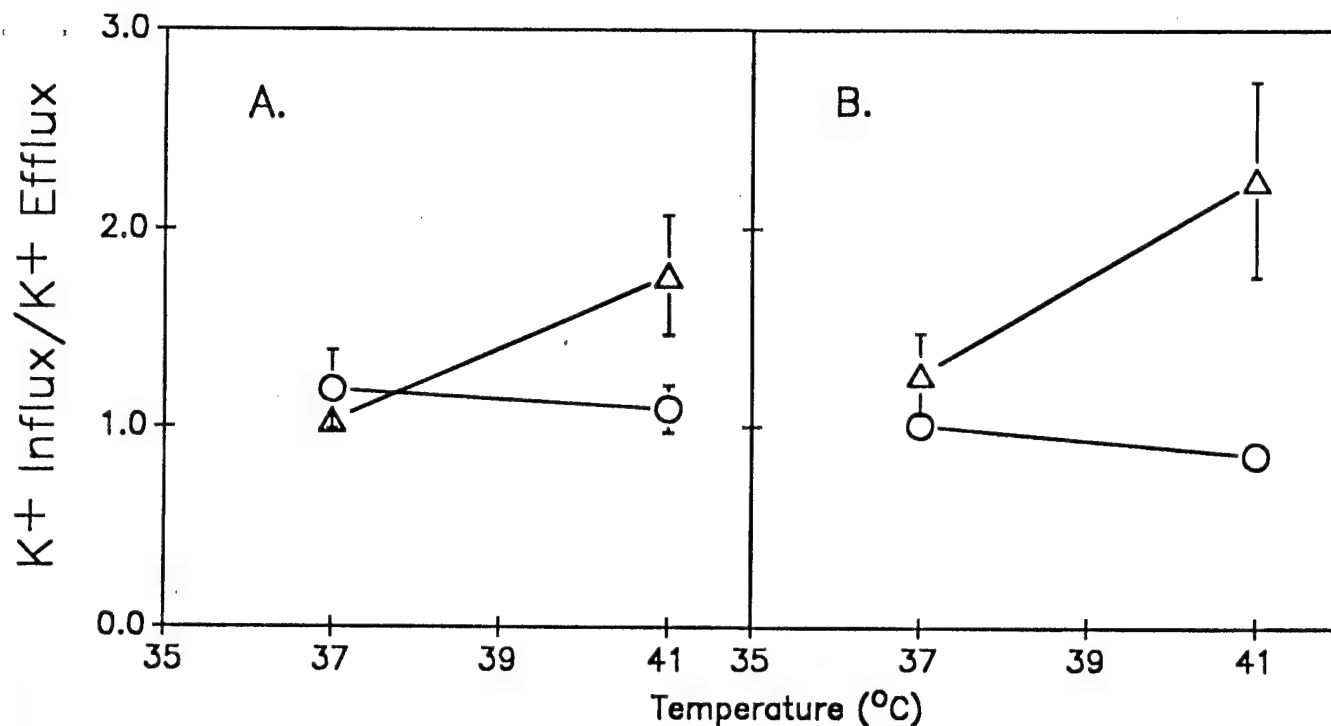


Fig. 7. Ratio of K⁺ influx to K⁺ efflux in guinea pig red cells in the presence of inhibition of K-Cl cotransport at 37°C and 41°C. K⁺ influx was measured by uptake of ⁸⁶Rb as described in Methods. K⁺ efflux was measured in parallel cells as in Figs. 6 and 7. Media contained 0.5 per cent albumin, but no ouabain or bumetanide. A. Circles, control cells; triangles, cells incubated in hypertonic medium. Points represent means \pm S.E. of five experiments. B. Circles, control cells; triangles, cells incubated with 25 nM calyculin. Points represent means \pm S.E. of four experiments.

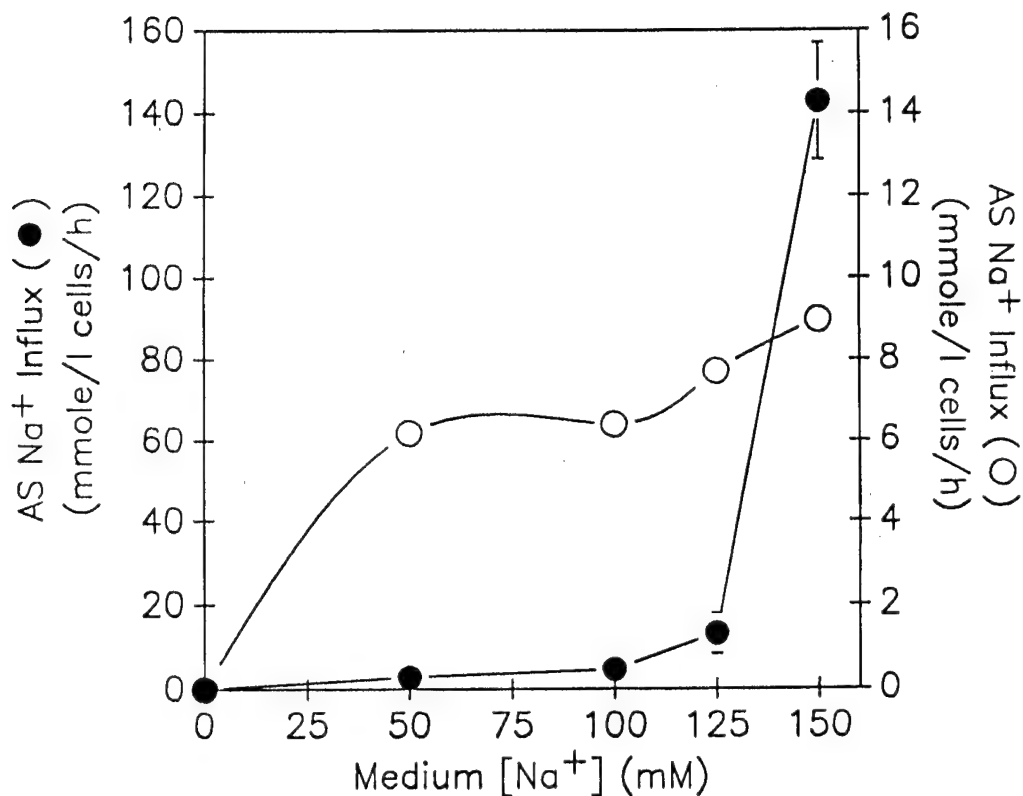


Fig. 8. Na-dependence of amiloride-sensitive (AS) Na⁺ influx in guinea pig red cells with cytoplasmic acidification. Guinea pig red cells were incubated in medium with NaCl (150 mM) or choline chloride (isotonic) or a combination of the two to produce the [Na⁺] concentration shown. Open circles: red cells from one guinea pig were incubated in medium with osmolarity raised to 450 mOsm by addition of choline chloride. The results duplicate those of Zhao & Willis (1993). Closed circles: Cells were equilibrated in medium with pH 6.0 as described in Methods and Materials. The cytoplasmic pH was during incubation was 6.4-6.7, and the medium pH was 7.4. Results shown are mean of 3 experiments \pm S.E. Note 10-fold difference in scales for two sets of data.

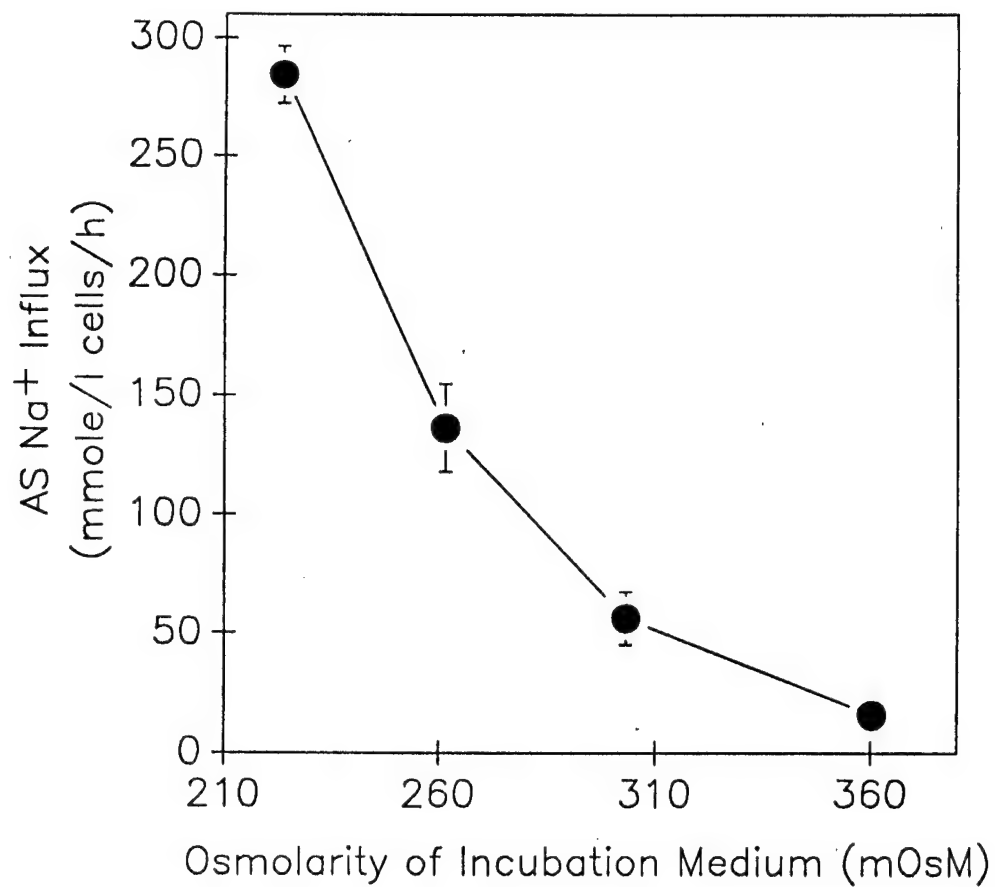


Fig. 9. Effect of osmolarity on amiloride-sensitive (AS) Na⁺ influx in acidified guinea pig red cells. Cells were acidified as in Fig. 1 and incubated in media with 100 mM NaCl (220 mOsM) whose osmolarity was adjusted upward by addition of NMDG. Temperature of incubation was 45°C (i.e., elevated to facilitate measurement of amiloride-sensitive Na⁺ influx at low [Na⁺]_o). Results shown represent means ± S.E. of 3 experiments.

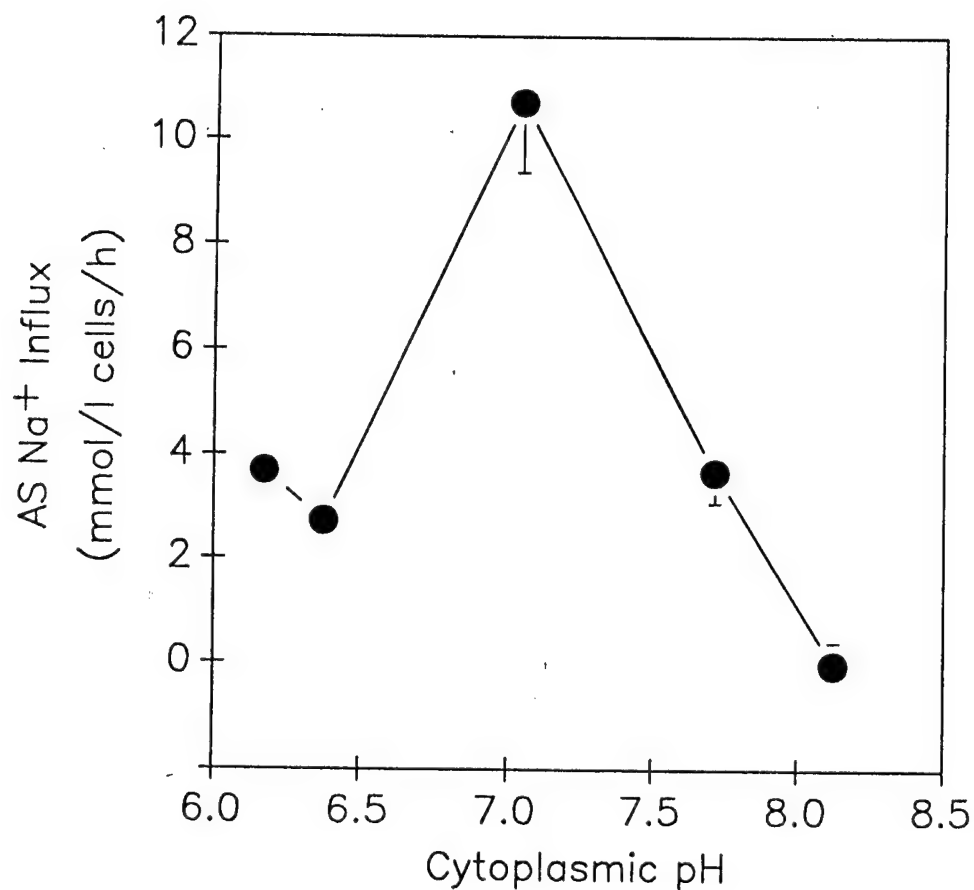


Fig. 10. Effect of cytoplasmic pH on amiloride-sensitive Na⁺ influx in hypertonically incubated guinea pig red cells. The pH of cells was adjusted by incubation in media with pH 6.0 - 8.5 and cytoplasmic pH was measured before and after incubation as described in Methods and Materials. Medium [Na⁺] was 125 mM and osmolarity was set to 530 mOsm by addition of NMDG. Temperature of incubation was 37°C. Results shown are means ± S.E. of 3 - 4 experiments.

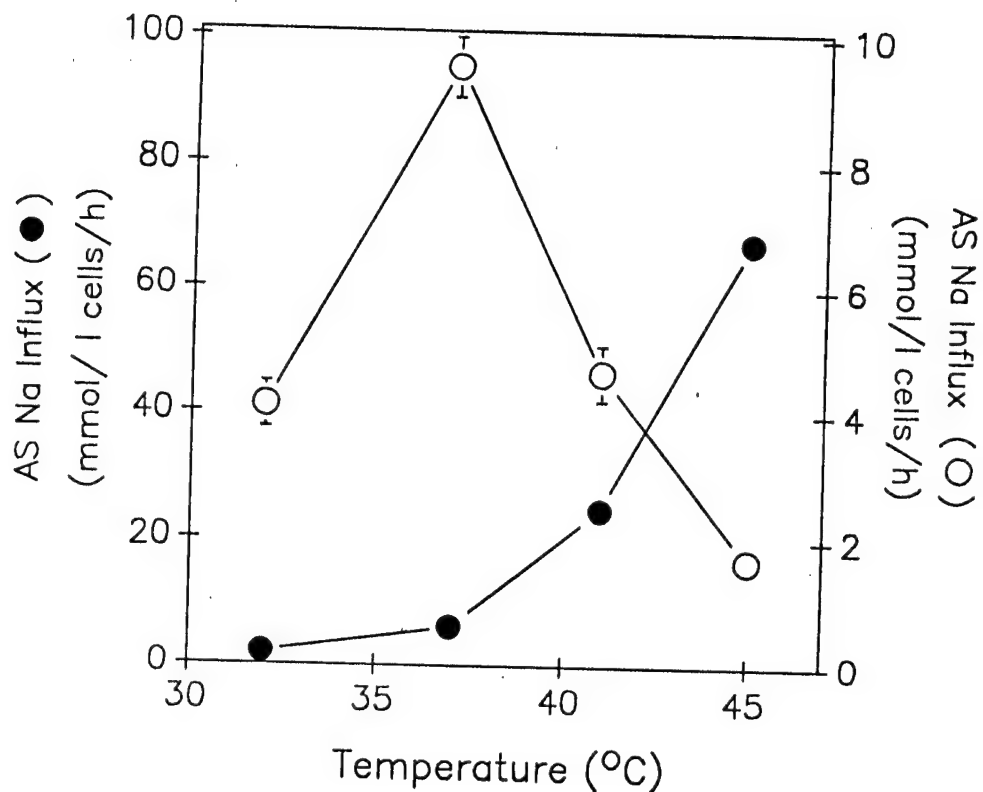


Fig. 11. Effect of temperature on two types of amiloride-sensitive (AS) Na⁺ influx. Based on results shown in Figs. 8 - 10, two types of preparation were subsequently used: "Type 1", in which cells were slightly alkalinized before being incubated in hyperosmotic medium and "Type 2", in which acidified cells were incubated in slightly hypotonic medium (125 mM NaCl, 260 mOsm). (To make media hypertonic in Type 1 preparations NMDG was added to yield 530 mOsm.) Open circles, Type 1 AS Na⁺ influx; closed circles, Type 2 AS Na⁺ influx. Note 10-fold difference in scales for two types of AS Na⁺ influx. Means \pm S.E. are shown for 3 - 8 experiments.

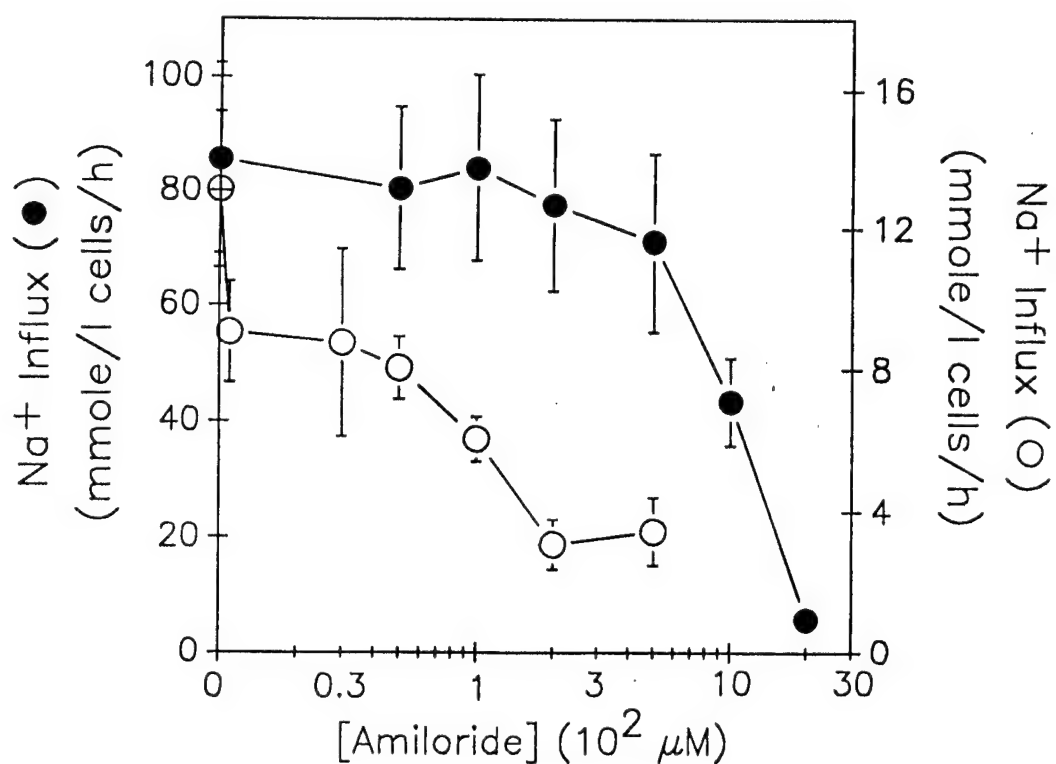


Fig. 12. Sensitivity to amiloride inhibition of two types of amiloride-sensitive Na^+ influx. Na^+ influx was measured in two types of preparation as defined in Fig. 11. Temperature of incubation was 37°C . Symbols are as defined in Fig. 4 and represent means \pm S.E. for three experiments. Note that although a log scale is used for amiloride concentrations, the left-most point for each group represents control cells in amiloride-free medium.

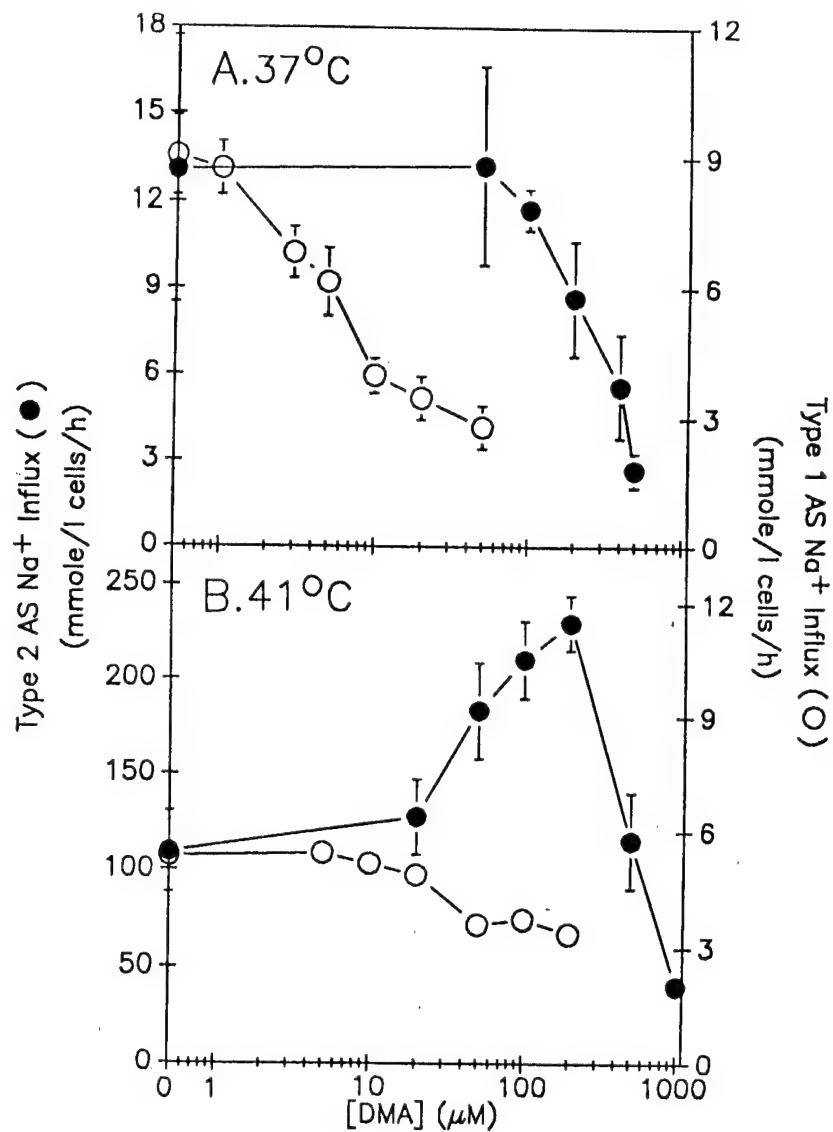


Fig. 13. Sensitivity of two types of AS Na⁺ influx to dimethylamiloride at two temperatures. Symbols and conditions are as described in Figs. 11 and 12. A. 37°C; B. 41°C. Open circles,, Type 1; closed circles, Type 2. Note difference in scales. Symbols represent means \pm S.E. of 3 experiments.

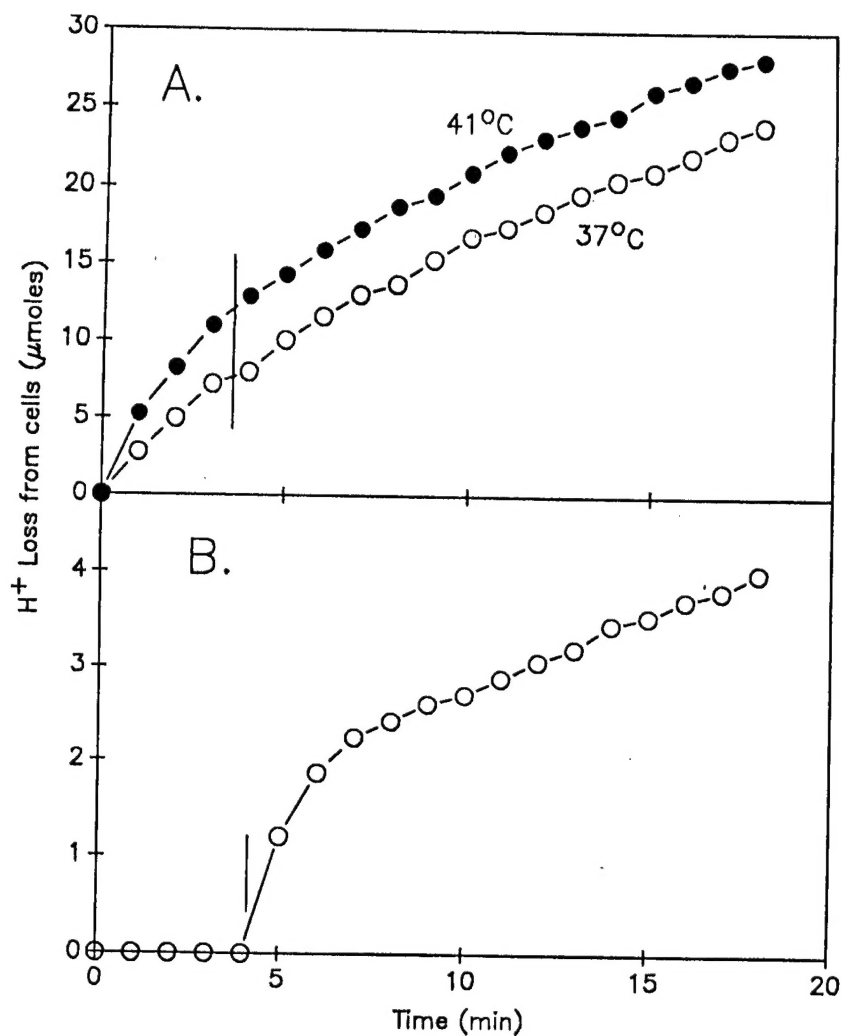


Fig. 14. H^+ loss from guinea pig red cells. Cells were incubated in lightly buffered medium and the loss of H^+ measured by titration with KOH as described in Methods and Materials. Points shown represent a single experiment representative of three similar experiments. A. Acidified cells. Initially cells were in isotonic medium. At the vertical line water was added to lower $[Na^+]_o$ to 125 mM (260 mOsm). Closed circles, 41°C; open circles, 37°C. B. Alkalized cells. At the vertical arrow sucrose was added to raise the osmolarity to 530 mOsm.

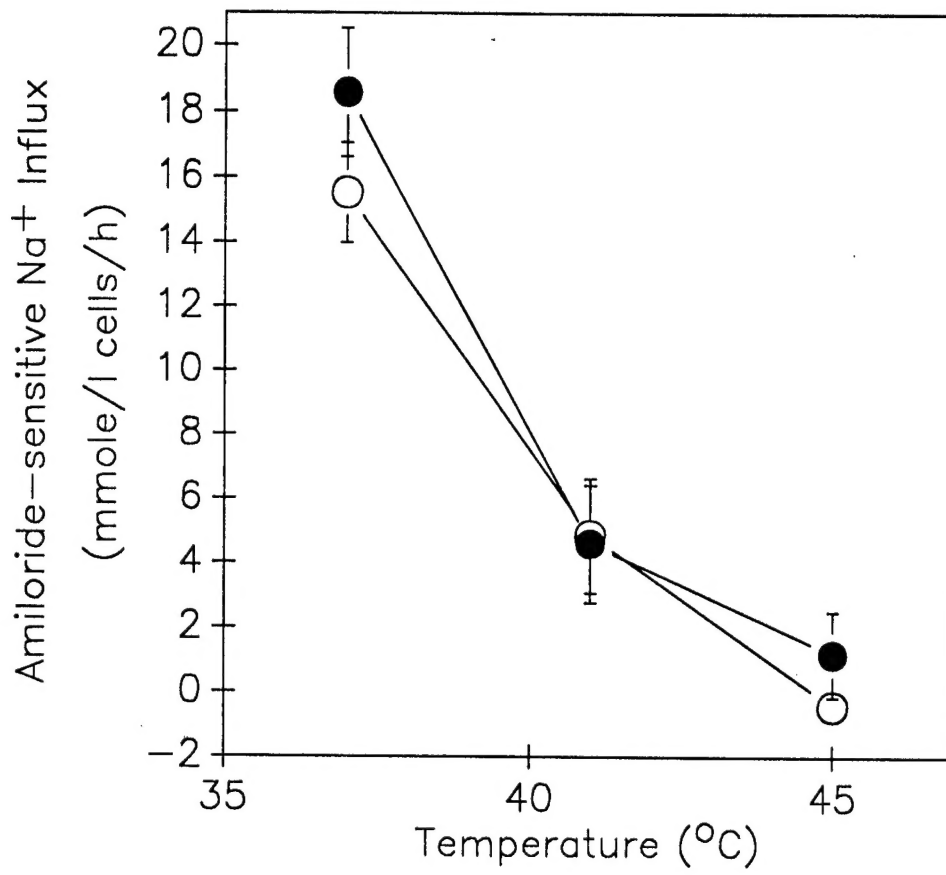


Fig. 15. Absence of effect of calyculin on shrinkage-activated AS Na⁺ influx. Cells were incubated in medium made hypertonic with NMDG to 530 mOsm. Results represent means of 4 experiments \pm S. E. Open circles, control; closed circles, 25 uM calyculin A.

BIBLIOGRAPHY

This project has so far resulted in three book chapters, two meetings abstracts and two papers submitted for publication. Text, figures and tables from the last two have been incorporated into this Final Report, where appropriate.

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- Willis, J. S. & Anderson, G. () Activation of K-Cl cotransport by moderate warming in guinea pig red cells. Submitted to *Journal of Physiology (London)*
- Ji, H. L. & Willis, J. S. () Two modes of amiloride-sensitive Na^+ influx in guinea pig red cells. Submitted to *Journal of Membrane Biology*

PERSONNEL

Persons who have been paid for effort on this grant:

John S. Willis (summer salaries)

Marimelia Villanueva

Gary Anderson